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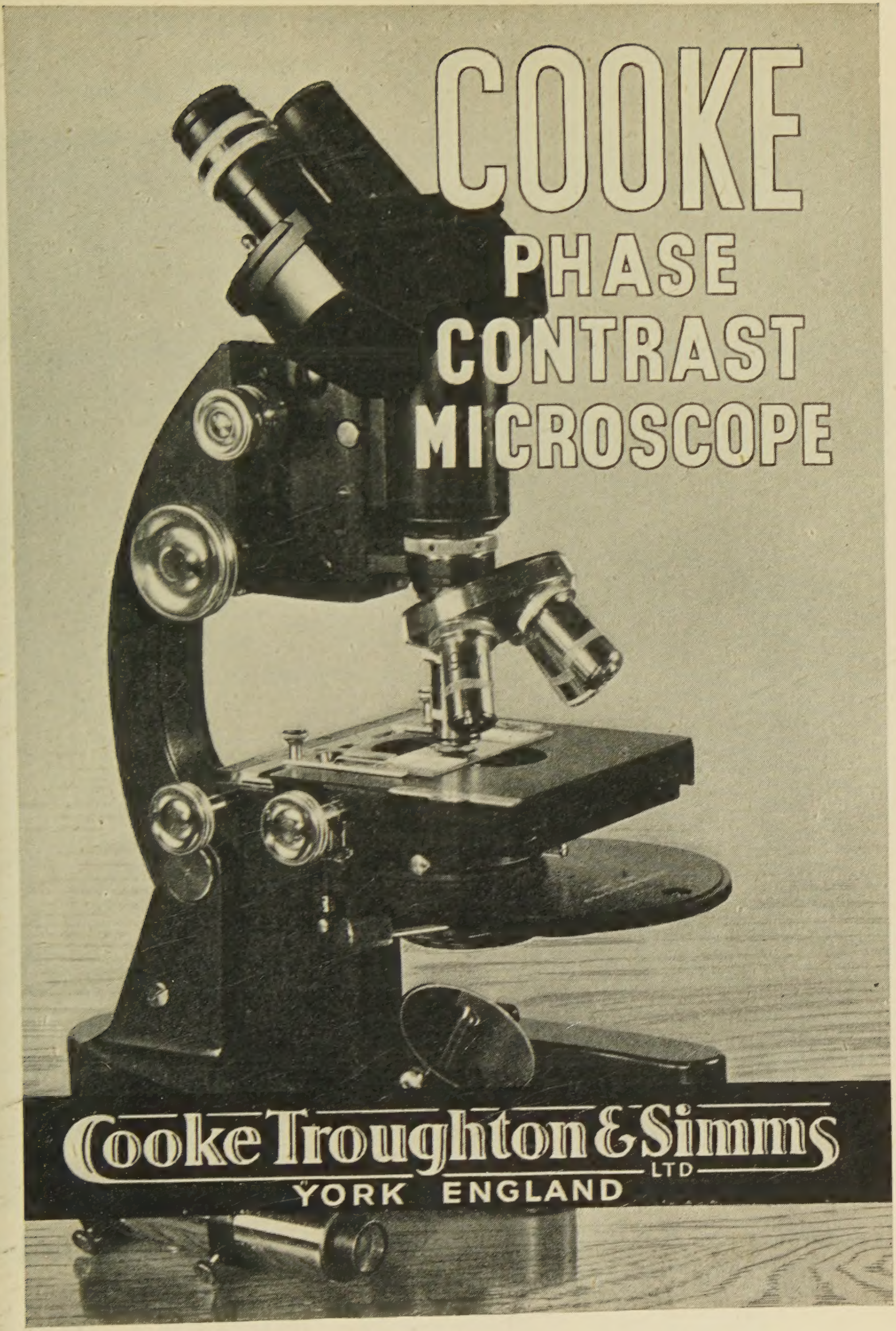
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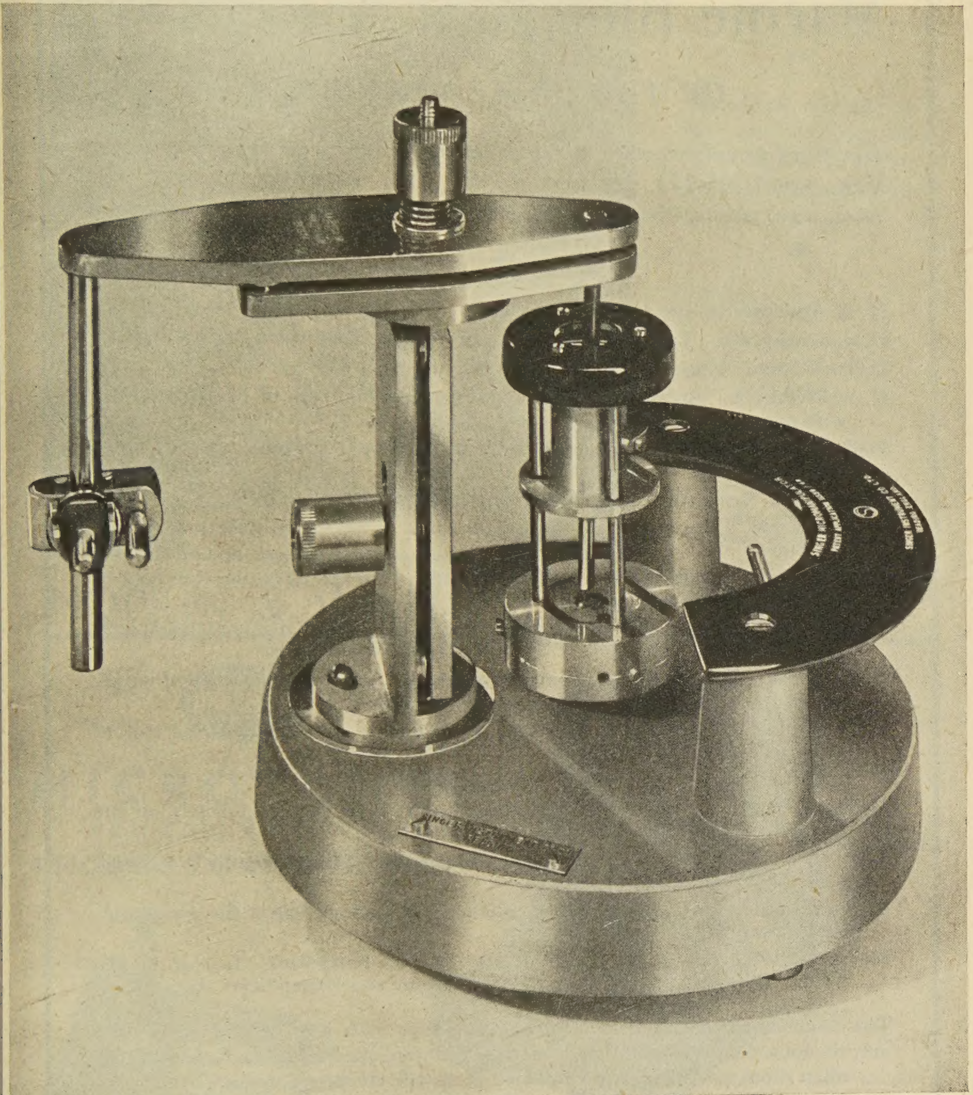

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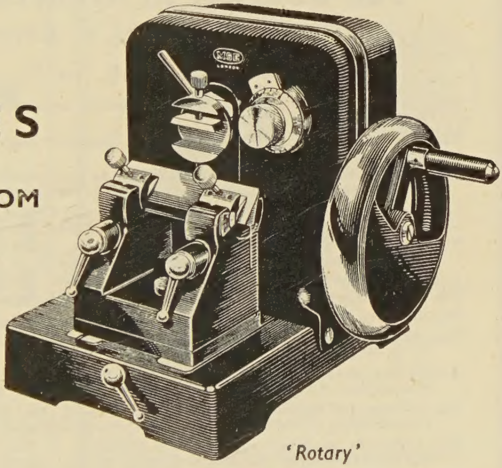

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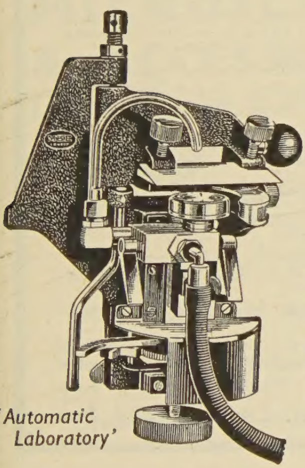

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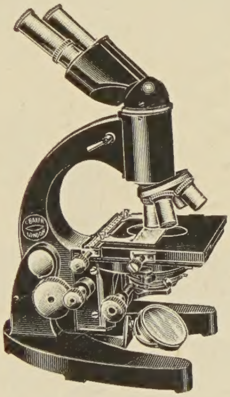

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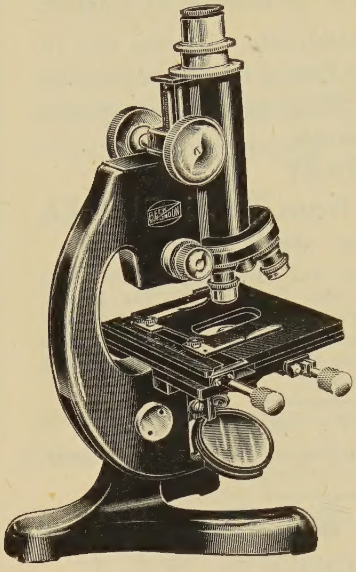

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Observations on the Branchial Crown of the Serpulidae (Annelida, Polychaeta)

BY

JEAN HANSON

(*From the Department of Zoology, Bedford College, University of London*)

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INTRODUCTION

DURING investigations on the blood systems of serpulids (Hanson, 1949) observations were made on the internal anatomy of the branchial crown. Previous accounts were found to be incomplete and in some respects inaccurate; so I decided to publish some of my observations. Thomas (1940) has given the most recent general description of the crown of a serpulid, *Pomatoceros*. Pruvot (1885) and Johansson (1927) showed, by studies on its innervation, that the crown of the Serpulimorpha represents the two prostomial palps of other polychaetes. It consists of filaments bearing pinnules, of the so-called 'palps', and, in most serpulids, of an operculum borne on a peduncle. It is generally recognized that the operculum and peduncle represent a modified filament (Zeleny, 1905; Segrove, 1941).

The following species were used: *Serpula vermicularis* L., *Hydroides norvegica* (Gunnerus), *Vermiliopsis infundibulum* (Philippi), *Pomatoceros triqueter* L., *Salmacina incrustans* Claparède, *Protula intestinum* (Lamarck), *Spirorbis corrugatus* (Montagu), and *S. militaris* (Claparède). *Pomatoceros* was obtained from Plymouth. The other species were studied at Naples. Most of the observations were made on sectioned specimens. The fixing solutions and staining methods I used are listed in my account of the blood system in the Serpulimorpha (Hanson, 1949).

OBSERVATIONS

Muscles

The muscles of the filaments (Text-fig. 1), pinnules (Text-fig. 6), 'palps'

[Quarterly Journal Microscopical Science, Vol. 90, part 3, September, 1949]

(Text-fig. 7), and opercular peduncle (Text-fig. 2) of *Pomatoceros* are all longitudinal.

In each *filament* are the following muscles:

(*a*) Two external branchial muscles (Thomas, 1940) situated in the two corners of the abfrontal side of the filament. Near to them are the two external branchial nerves.

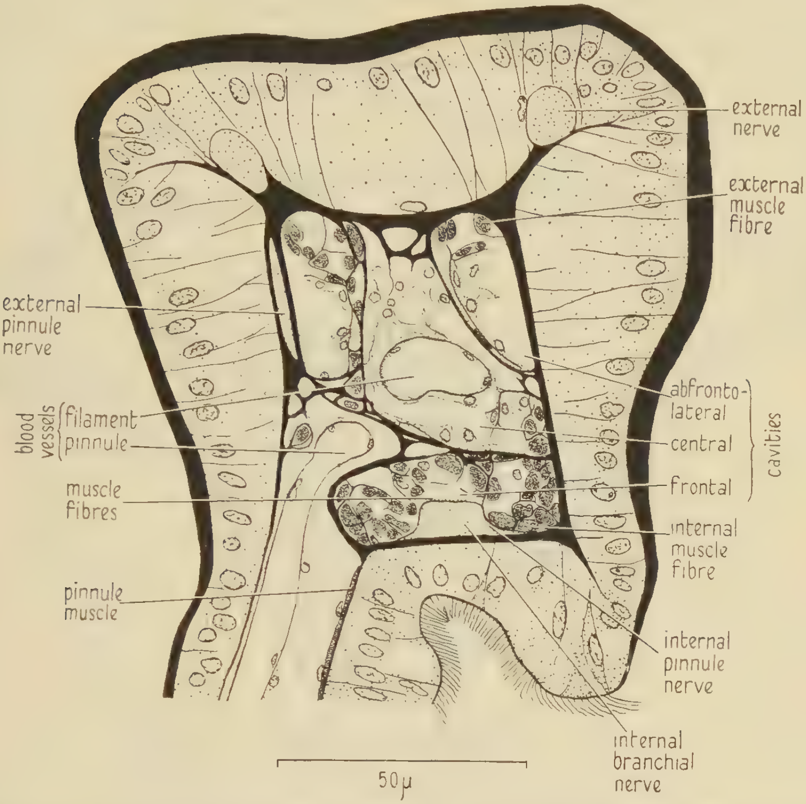
(*b*) Two internal branchial muscles (Thomas) situated on the frontal side of the filament. The fibres of these muscles are grouped round the internal branchial nerve. Most of them lie on each side of the nerve, but some are abfrontal to it.

(*c*) A single layer of small muscle-fibres situated on the abfrontal surface of the internal branchial nerve. They are not present on the external branchial nerves or on any other nerves except the internal peduncular nerve.

The *peduncle* (Text-fig. 2) is triangular in cross-section, and contains a single group of muscle-fibres situated in the apical angle (frontal) and along the two sides. The fibres do not enter the operculum, but terminate at the top of the peduncle. Here they are attached to the basement membrane of the epidermis which roofs the peduncle, except for a small aperture through which the blood-vessel and its surrounding connective tissue and the three opercular nerves enter the operculum. McIntosh (1922–3) stated that the muscles of the peduncle are 'chiefly transverse', and concluded: 'The thrusting out of the operculum—if such happens—is thus voluntary, the withdrawal and retention more or less involuntary.' I have found that the peduncular muscle, like the branchial muscles, consists of longitudinal fibres, the contraction of which will draw the operculum towards the centre of the crown when the animal withdraws into its tube. When the crown is expanded the peduncle is sometimes held out nearly at right angles to the long axis of the body.

Each *pinnule* (Text-fig. 6) and '*palp*' (Text-fig. 7) has only a few muscle-fibres, situated just inside the basement membrane of the frontal epidermis.

A pair of internal and a pair of external branchial muscles are present in each filament of *Serpula* (Text-fig. 3), *Hydroides*, *Vermiliopsis*, *Salmacina*, and *Spirorbis militaris*. As in *Pomatoceros*, the external branchial muscles are less well developed than the internal branchial muscles, but they are more reduced in these serpulids than in *Pomatoceros*. In *Protula* and *Spirorbis corrugatus* external branchial muscles could not be found, but internal branchial muscles are present. In their internal structure the peduncles of both functional and reserve opercula of *Serpula* and *Hydroides* are very similar to filaments; the internal and external peduncular muscles have the same appearance as their counterparts in the filaments. The arrangement of the muscles in the pinnules and 'palps' of *Serpula*, *Hydroides*, and *Vermiliopsis*, and in the pinnules of *Protula* and *Spirorbis militaris* is the same as in *Pomatoceros*. Muscle-fibres on the abfrontal surface of the internal branchial nerve have been found in the following serpulids besides *Pomatoceros*: *Serpula*, *Hydroides*, *Vermiliopsis*, *Protula*, and *Spirorbis militaris*. Their function is not known. Comparable muscles of unknown function occur on the dorsal surface of the ventral nerve cord of *Nereis* and other errant polychaetes (Prenant, 1929), and on the lateral nerves of pelagic nemerteans (Coe, 1926). Probably other cases exist.



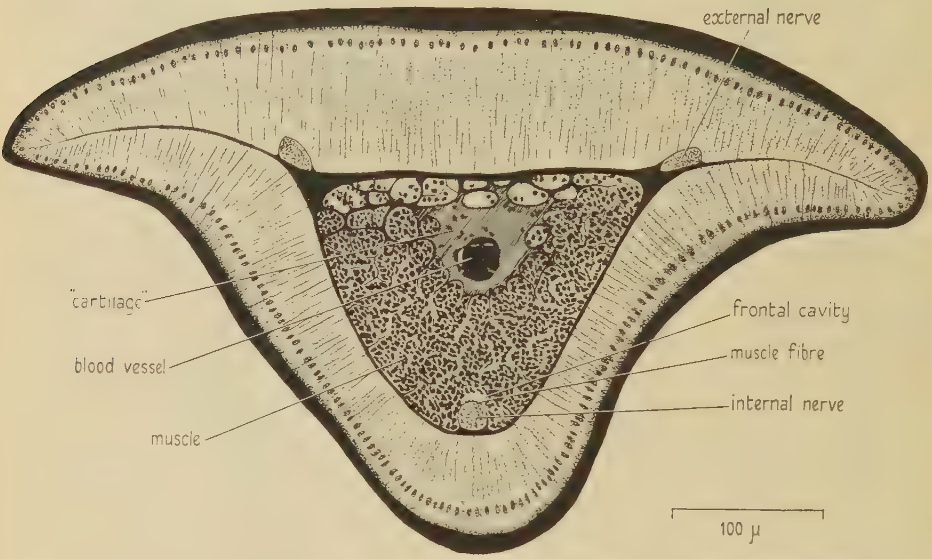
TEXT-FIG. 1. Transverse section through filament of *Pomatoceros triqueter*, and longitudinal section through base of one pinnule.

Nerves

In the *peduncle* and each *filament* of *Pomatoceros* there are three nerves (Thomas, 1940). Two are situated in the abfrontal corners (the external peduncular and external branchial nerves) and the third on the frontal face (internal peduncular and internal branchial nerves). The abfrontal nerves lie outside the basement membrane of the epidermis. The frontal nerve lies just inside the basement membrane. On its abfrontal surface is a layer of small muscle-fibres (see above) and a small well-defined canal, part of the system of cavities in the crown (p. 229). No muscles or canals accompany the abfrontal nerves.

All three branchial nerves send branches into the *pinnules*. The branches of the internal and external branchial nerves are respectively the frontal and abfrontal nerves of the pinnules (Text-figs. 1 and 6). The nerves of the pinnules lie outside the basement membrane of the epidermis. Thomas found the frontal pinnule nerves, but has stated that abfrontal nerves are absent.

At the top of the *peduncle* the muscles and canal accompanying the internal peduncular nerve terminate, and the two external peduncular nerves come to lie just inside the basement membrane of the epidermis. Each of the three nerves now gives off two small branches which I have been able to trace for a short distance only. The three nerves enter the operculum through the small gap by which the inside of the operculum communicates with the inside of the peduncle. Within the operculum all three nerves pass to the outside of the basement membrane. They give off many small branches, and can be traced as far as the rim round the top of the operculum.



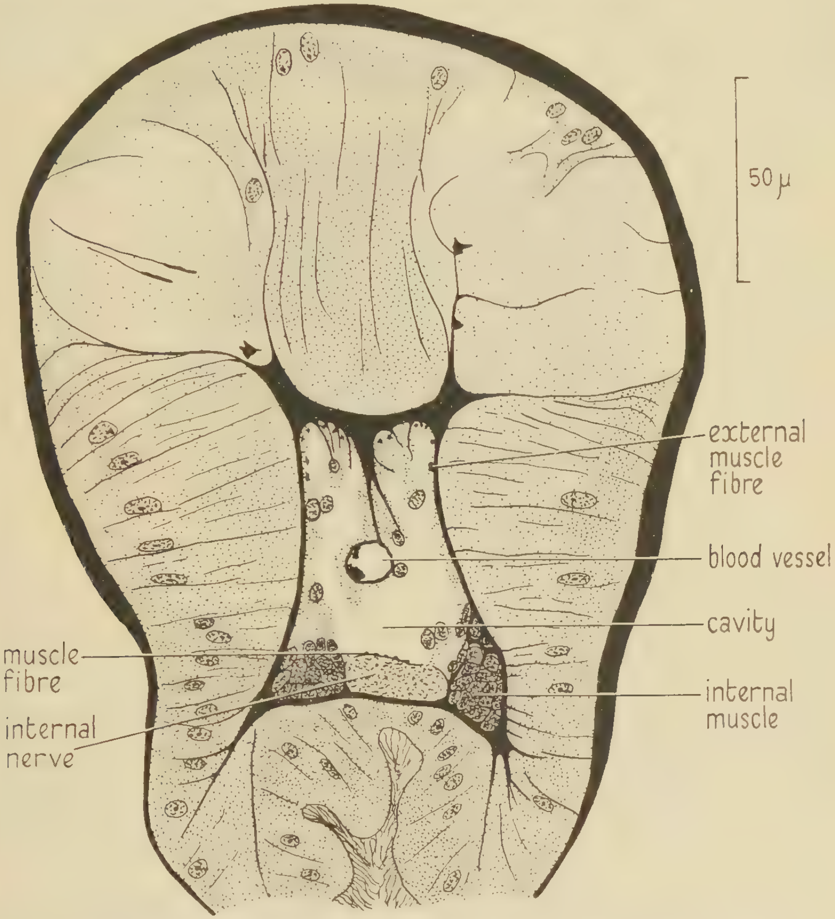
TEXT-FIG. 2. Transverse section through opercular peduncle of *Pomatoceros triqueter.*

The internal and external branchial nerves of the following serpulids are like those of *Pomatoceros*: *Hydroides*, *Vermiliopsis*, *Protula*, *Salmacina*, *Spirorbis corrugatus*, and *S. militaris*. No external branchial nerves could be found in *Serpula*. All my material was stained with 'Azan', however; different histological methods might reveal them, although they are distinct in Azan-stained sections of other serpulids. The internal and external peduncular nerves of *Hydroides* are like the corresponding nerves in the filaments. Both internal and external pinnule nerves have been found in *Hydroides*; internal pinnule nerves are present in *Vermiliopsis*, *Protula*, and *Spirorbis militaris*, but external pinnule nerves could not be found. Meyer (1888) was unable to find external pinnule nerves in *Eupomatus lunuliferus* (= *Hydroides lunulifera*) or *Psygmobranchus protensus* (= *Protula tubularia*), but Faulkner (1930) saw them in *Filograna implexa*.

The functional significance of the double nerve-supply to the serpulid crown is not known.

Skeleton

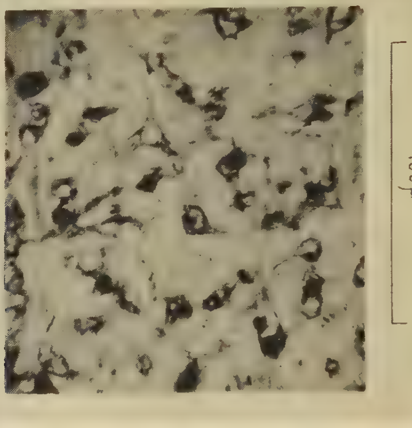
The internal skeleton of the *filament* of *Pomatoceros* consists of fibres enmeshing the muscle-fibres of the internal and external branchial muscles,



TEXT-FIG. 3. Transverse section through filament of *Serpula vermicularis.*

and of sheets, namely, the basement membrane of the epidermis and the skeletal coat of the blood-vessel wall (Hanson, 1949). Both the fibres and the sheets give the staining reactions of collagen. The fibres, and the basement membrane of the epidermis, but not the skeletal coat of the vessel wall, enclose reticular fibres. No elastic fibres are present.

In the *peduncle*, as in the filaments, the muscle-fibres are enmeshed by connective tissue-fibres. On the abfrontal surface of the peduncular muscle these fibres pass into the ground substance of a cartilage-like tissue which occupies the abfrontal part of the peduncle and fills the operculum, except for the space taken by the spiral opercular blood-vessel (Hanson, 1949). The ground substance gives the staining reactions of collagen, and is penetrated by reticular fibres and by a network of cytoplasm containing nuclei (Text-fig. 4). Ewer and Hanson (1945) have found that the ground substance reacts negatively to mucicarmine, thionin, resorcin-fuchsin, orcein, and safranin, all of which stain the ground substance (mucoprotein) of mammalian cartilage in a characteristic ('positive') manner. The cartilage-like tissue of *Pomatoceros* is very similar to the 'perichondrial' part of the branchial skeleton of *Sabella pavonina*, as described by Nicol (1930). Ewer and Hanson have found that the 'perichondrium' of *S. spallanzanii* reacts negatively to mucoprotein stains, but that the thick walls of the large vacuolated cells which make up the axial part of the skeleton are coloured by these stains in the same manner as mammalian cartilage (and sabellid mucous cells); they presumably contain a mucoprotein. Some similar results were obtained by Nowikoff (1912).



TEXT-FIG. 4. Photomicrograph of section through 'cartilage' in operculum of *Pomatoceros triqueter*. Flemming. Iron haematoxylin.

In the *pinnule* and '*palp*' of *Pomatoceros* and other serpulids, the internal skeleton is represented only by the basement membrane of the epidermis and the skeletal coat of the vessel wall.

In the filaments of serpulids other than *Pomatoceros*, external branchial muscles either appear to be absent or are represented only by a few fibres lying near the basement membrane of the abfrontal epidermis (Text-fig. 3). There are no connective tissue-fibres enmeshing these muscle-fibres. The skeleton associated with the internal branchial muscles is as well developed as in *Pomatoceros*. In *Hydroides*, a single stout fibrous sheet stretches from the skeletal coat of the blood-vessel wall to the medial line of the basement membrane of the abfrontal epidermis (figured by McIntosh, 1926). *Serpula*

(Text-fig. 3) and *Protula* are similar. In *Vermiliopsis* the vessel is anchored by several such fibrous sheets. In *Salmacina*, *Spirorbis militaris*, and *S. corrugatus* the vessel lies on the basement membrane of the abfrontal epidermis. In *Pomatoceros* the vessel lies in the centre of the filament, and fine fibres anchor it mainly to the skeleton around the external and internal branchial muscles.

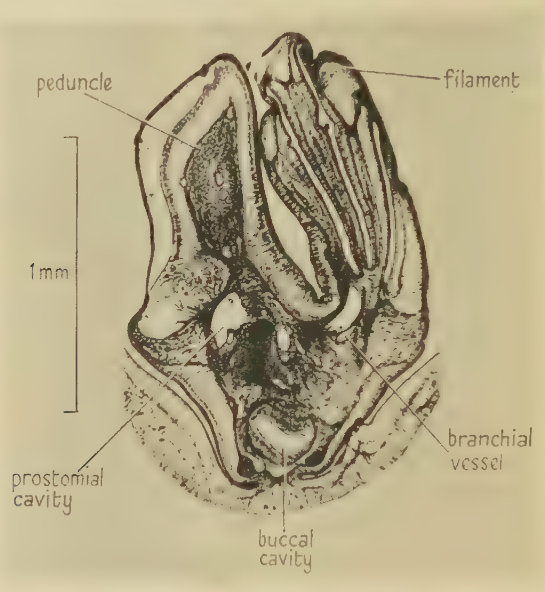
The branchial skeleton of *Sabella* consists of large, vacuolated thick-walled cells surrounded by a cartilage-like sheath (Nicol, 1930). I have seen a similar skeleton in *Potamilla* sp. and *Dasychone lucullana* Delle Chiaje. No serpulid has yet been found to possess a skeleton like this. Large vacuolated cells with strong walls are commonly found in the *epidermis* of the serpulid crown, e.g. in *Filograna implexa* (Faulkner, 1930) and *Protula* (= *Salmacina*) *dysteri* (Huxley, 1855). In *Pomatoceros* their walls are not as thick as those of the axial part of the sabellid skeleton, and do not give the same staining reactions.

The internal skeleton in the peduncles of the functional and reserve opercula of *Serpula* and *Hydroides* is very similar to that in their filaments. 'Cartilage', of the same type as in *Pomatoceros*, is confined to the opercula which it fills, except for the spaces taken by the branched blood-vessels. The 'cartilage' in the reserve opercula has a much denser cell population than in the functional opercula. The 'cartilage' of *Serpula* and *Hydroides* was described by Örley (1884) and Zeleny (1905), respectively.

Cavities

The cavities in the prostomium and peristomium of *Pomatoceros* have processes extending into the base of the crown, where they end blindly. I have found that they are not, as previously stated (McIntosh, 1918; Thomas, 1940), continuous with the cavities in the filaments. The nature of all these cavities is obscure. 'It is difficult to determine whether the cavity in this region, which represents the head and collar segment, is of blastocoelic or coelomic origin . . .' (Segrove, 1941). Observations now to be reported have been made on the arrangement of the cavities in the peristomium, prostomium, and base of crown of *Pomatoceros*, and in the filaments, pinnules, 'palps', and peduncle of *Pomatoceros* and other serpulids.

The posterior septum of the cavity of the peristomium of *Pomatoceros* joins the alimentary canal at the junction between the oesophagus and the stomach. The main part of the peristomial cavity extends forwards as far as the level of the cerebral ganglia, and two processes from it accompany the two branchial blood-vessels to the base of the crown. I have found that the ventral mesentery extends throughout the peristomium as far as its front wall, and does not end behind the peristomium as stated by Thomas (1940). The peristomial part of the dorsal mesentery extends forwards only as far as the level of the transverse canal joining the two thoracic nephridia. Here the tissue of the dorsal mesentery becomes detached both from the roof of the peristomial cavity and from the wall of the oesophagus, and becomes a sheath around the dorsal blood-vessel. Posteriorly, the peristomial cavity consists of two halves, right and left, separated by the dorsal and ventral mesenteries. Anteriorly it is irregularly subdivided by the ventral mesentery and by strands of tissue stretching from the body-wall to the wall of the oesophagus. The ciliated funnels of the two thoracic nephridia open laterally into the peristomial cavity in this anterior region. If, as Meyer's work on *Protula tubularia* (Meyer, 1888) seems to indicate, these ciliated funnels are coelomoducts, the fact that they open into the peristomial cavity suggests that this cavity is



Text-fig. 5. Photomicrograph of obliquely transverse section through base of crown of *Pomatoceros triqueter*.

coelomic, and the presence of dorsal and ventral mesenteries strengthens this supposition.

The cavity of the prostomium is confined to the dorsal part of the animal; it extends posteriorly for a short distance behind the cerebral ganglia, and anteriorly into the base of the crown. It is separated into two halves, right and left, by a septum enclosing the common median duct of the nephridia.

The base of each branchial blood-vessel lies in a process of the peristomial cavity, passing forwards between the dorsal and ventral roots of the circum-oesophageal nerve commissures, and ending blindly before the branchial vessel gives off the first of the filament vessels. Also accompanying each branchial vessel is one-half of the cavity of the prostomium (Text-fig. 5); it extends round the base of the crown and ends blindly where the branchial vessel enters the most ventral filament. The cavities of the prostomium and peristomium do not communicate with each other. On the left side each sends a blind-ending branch into the base of the peduncle; they accompany the opercular blood-vessel, which is a branch of the left branchial vessel. The peristomial and prostomial cavities have no connexion with the cavities in the filaments.

McIntosh (1918) noticed cavities in the filaments of *Pomatoceros*, and thought it probable that they communicate with the 'coelomic' space around the branchial vessel, although he was not able to find the communication. He did not describe the derivation of this 'coelomic' space, but from his figures it is clear that he was referring to the anterior part of the prostomial cavity. Thomas (1940) has stated that the 'coelom' of the peristomium 'sends a pair of extensions passing between the dorsal and ventral roots of the oesophageal connectives giving branches into the filaments . . . '. Her figure of a transverse section through one of the filaments (Fig. 8) suggests that she has mistaken as a coelomic channel the space which usually appears in fixed specimens between the wall of the vessel and the mass of coagulated blood lying in its centre. It may be concluded that she has not demonstrated any extension of the peristomial cavity into the filaments. Meyer (1888) thought that the 'secundäre Leibeshöhle des Kopfmundsegmentes' of *Hydroides lunulifera* and *Protula tubularia* is continuous with the channel surrounding the vessel in the centre of each filament. Faulkner (1930) has stated that in *Filograna implexa* the prostomial cavity sends a process on each side into the base of the crown, with branches into each of the filaments.

Each *pinnule* and '*palp*' of *Pomatoceros* contains a large central cavity lined by a thin epithelium lying on the basement membrane of the epidermis. In the frontal part of the lining are situated the longitudinal muscles; fused to the abfrontal lining is the blood-vessel.

The cavities of the pinnules are branches of the central cavities of the *filaments* (Text-fig. 1). Each filament contains four cavities, which extend without interruption along the whole of its length.

(*a*) A central channel surrounding the blood-vessel.

(*b*, *c*) A pair of abfronto-lateral channels situated between the lateral epidermis and the external branchial muscles. These are probably the 'coelomic' cavities of McIntosh (1918).

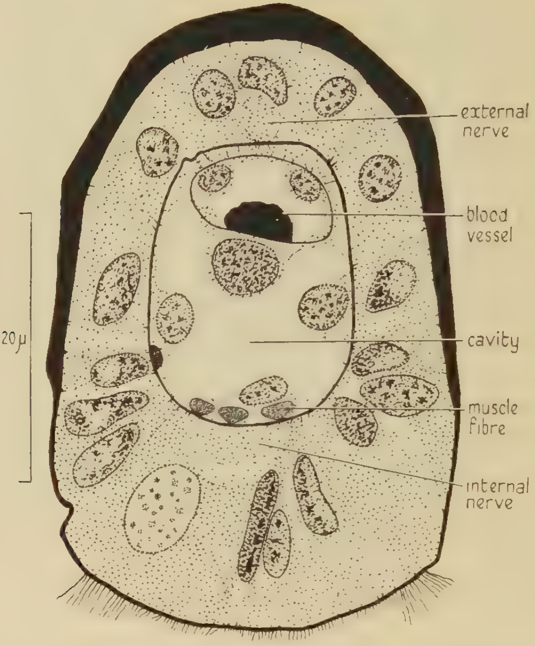
(*d*) A small channel situated on the abfrontal face of the internal branchial nerve, and separated from the central cavity by some of the fibres of the internal branchial muscles and their surrounding connective tissue.

The *operculum* contains no cavities. The first three cavities of the filaments are not represented in the *peduncle*, but the internal peduncular nerve is accompanied by a small channel like that of the internal branchial nerve (Text-fig. 2).

In the following serpulids the pinnule cavity is like that of *Pomatoceros*, but in the filaments there is only a single cavity, which is large and occupies most of the interior: *Serpula* (Text-fig. 3), *Hydroides*, *Vermiliopsis*, *Protula*, and *Spirorbis militaris*. The cavities in the peduncles of the functional and reserve opercula of *Serpula* and *Hydroides* are like filament cavities.

The 'palps'

The so-called 'palps' of *Pomatoceros* are two short processes situated between the dorsal lip and the most dorsal filaments of the two halves of the crown. Studies on the feeding method (Johansson, 1927; Thomas, 1940) have shown that the 'palps' are concerned with the rejection of unwanted particles. Except in a few minor points, the structure of a 'palp' of *Pomatoceros* is identical with that of a pinnule.

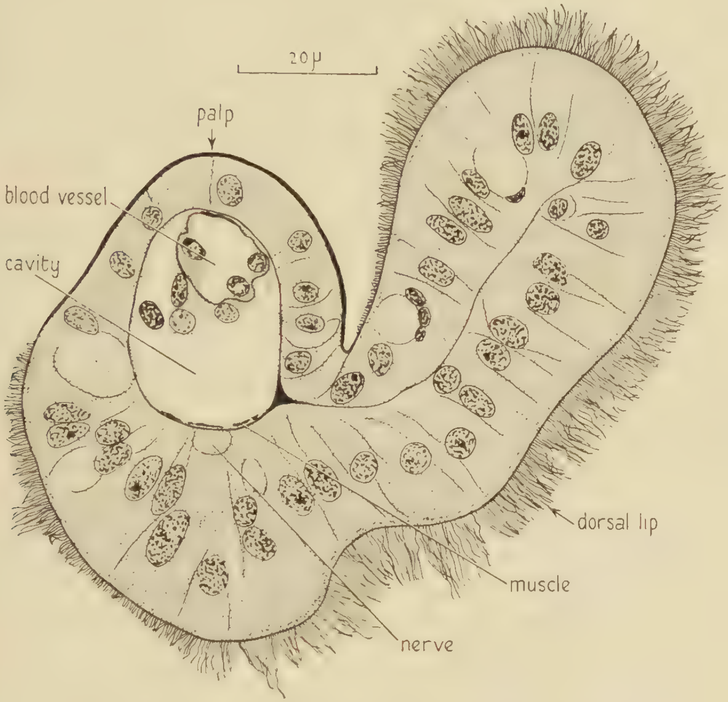


TEXT-FIG. 6. Transverse section through pinnule of *Pomatoceros triqueter*.

At its base the 'palp' is fused both with the adjacent filament and with the dorsal lip. A short distance from the base it becomes detached from the filament, but remains attached to the lip, from which it becomes free only at its tip. The lip consists of two epithelia, whose basement membranes are fused except where blood-vessels and nerves are situated between them. As Thomas has shown, these nerves arise from a branch of the internal branchial nerve of the most dorsal filament of each side. The lip vessels have no connexion with the 'palp' vessels, and are supplied with blood from the circum-oesophageal vessels (Hanson, 1949).

The epidermis of the 'palp' (Text-fig. 7) rests on a basement membrane inside which is the central cavity, lined by a thin epithelium as in a pinnule. The disposition of the muscles and blood-vessel of the 'palp' is the same as in a pinnule. The vessel is a branch of the vessel of the adjacent filament. As Thomas has shown, the internal branchial nerve of this filament gives off a branch which innervates both the 'palp' and the dorsal lip. In the 'palp' the nerve is situated at the base of the frontal epidermis; it corresponds to the internal pinnule nerve. I have been unable to find an abfrontal nerve in the 'palp', although one is present in the pinnule.

The very close structural resemblance between 'palp' and pinnule suggests that the 'palp' may be a modified pinnule (compare Text-figs. 6 and 7). This is supported by the fact that its blood-vessel and nerve are branches of those of the adjacent (most dorsal) filament. Moreover, the arrangement of pinnules at the base of this dorsal filament is atypical. In other filaments the pinnules are arranged in pairs from the base to the tip. In each of the most dorsal filaments there are two unpaired pinnules situated below the most basal paired pinnules, and both arise from the side adjacent to the 'palp'. Segrove (1941) has noticed that, at an early stage in development, the basal pinnule of the most dorsal filament 'is unpaired and arises on the median side of the filament'.



Text-fig. 7. Transverse section through 'palp' and accompanying part of dorsal lip of *Pomatoceros triqueter*.

Segrove's account of the early development of the branchial crown, however, does not lend any support to the view that the 'palp' is a modified pinnule. He has found that the crown first appears as six primary branchial processes, three on each side of the head. The most dorsal pair develops into the 'palps', and the others into filaments, except one on the left side which becomes the operculum and its peduncle. At first, the ciliation of the primary branchial processes is like that of adult pinnules. The 'palps' retain this type of ciliation, do not grow as rapidly as the other processes, and do not develop pinnules. The other processes acquire the ciliation of filaments when their pinnules develop. This account of the external appearance of the developing crown suggests that the 'palps' represent filaments which have retained their juvenile ciliation. However, it is possible that they are precociously developed pinnules belonging to the adjacent filaments. It is clear that information about the internal anatomy of the crown of very young specimens is needed to elucidate the morphological nature of the 'palps'. The development of the 'palps' of the sabellid *Branchiomma* (Wilson, 1936) is similar to that of *Pomatoceros.*

The 'palps' of *Serpula*, *Hydroides*, and *Vermiliopsis* are like pinnules in structure, and their blood-vessels are branches of the vessels supplying the most dorsal filaments in the crown.

Part of this investigation was made at the Zoological Station of Naples. I am grateful to the staff of the Station, to the British Association for the Advancement of Science for the use of its Table, and to the University of London for a grant towards travelling expenses.

SUMMARY

1. This paper records various observations supplementing, and in some cases correcting previous accounts of the internal structure of the branchial crown in *Pomatoceros triqueter*, *Serpula vermicularis*, *Hydroides norvegica*, *Vermiliopsis infundibulum*, *Salmacina incrustans*, *Protula intestinum*, *Spirorbis corrugatus*, and *Spirorbis militaris.*

2. The muscles in the opercular peduncle of *Pomatoceros* are longitudinal.

3. A single layer of small longitudinal muscle-fibres, of unknown function, has been found on the abfrontal face of the internal branchial and internal peduncular nerves of most of these serpulids.

4. External branchial muscles are well developed in *Pomatoceros*, reduced in *Serpula*, *Hydroides*, *Vermiliopsis*, and *Spirorbis militaris*, and apparently absent in *Protula* and *Spirorbis corrugatus.*

5. The pinnules of *Pomatoceros* and *Hydroides*, like filaments and opercular peduncles, have a double innervation.

6. The internal skeleton of the serpulid crown consists of sheets and strands of connective tissue-fibres and, in the opercula, of a cartilage-like tissue with a ground substance giving the staining reactions of collagen. The serpulid branchial skeleton is compared with that of sabellids, and the chemical nature of these skeletons is discussed.

7. Extensions of the prostomial and peristomial cavities accompany the branchial blood-vessels into the base of the crown, but have no connexion with the cavities in the branches of the crown. The arrangement of the latter

is described. The nature of all these cavities, whether coelomic or blastocoelic, is obscure.

8. The 'palps' of *Pomatoceros*, *Serpula*, *Hydroides*, and *Vermiliopsis* closely resemble pinnules. The blood-vessel and nerve of the 'palp' of *Pomatoceros* are branches of those in the adjacent filament. It is suggested that the serpulid 'palp' is a modified pinnule.

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The Polymorphic Transformations of *Obelia*

BY

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Part I. Stolonic growth and Perisarc

Part II. Hydranth development

Part III. Gonangium and Medusae

INTRODUCTION

THE following account is a portrayal of a single hydroid species, *Obelia commissularis*, as a living organism in all of its manifestations.

Obelia has long been established as the text-book type of a marine hydroid, illustrating the colonial condition and so-called alternation of generations. In the present century it has been the subject of several special studies. Huxley and de Beer (1923) have given a detailed account of the process of hydranth resorption. Lund (1921–6), in a series of papers, correlated hydranth formation and regeneration with electrical polarities. Commencing in 1933 and culminating in a general review started in 1943, Hammett and his co-workers published numerous papers on the role of amino-acids and other factors upon the several phases of growth as defined by them. *Obelia geniculata* was employed as the test organism, and while the approach is dynamic and numerous observations on the formation of the living hydranth are reported (Hammett, 1943; Hauscha, 1944), the 'concern here is not with organism as organism; but with organism as test material suitable for evaluation of the interdependencies of growth'. Much of the value of this work depends upon the validity of the various abstractions made of the developmental process as a whole. Since our own emphasis is on *Obelia* as an organism, it is unnecessary to discuss the question further at this time. In later papers Hammett (1945–6) presents statistical analyses of the condition of the hydranth population of thousands of colonies, undoubtedly a herculean labour but one that throws into sharp relief the difficulty, if not the impossibility, of analysing individual behaviour in terms of statistical averages, for an organism persists in being primarily organismal in spite of anything an investigator may have in mind.

Studies of *Obelia* are included in the older work of Weismann, in his monograph (1883) on the origin of the sex cells in hydroids. Weismann's illustrations are unequalled in beauty and accuracy, but they were interpreted in an extremely static manner remote from any concept of a living organism,

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and with a misleading preconception of the continuity and nature of germ plasm, a theory ostensibly but not actually based upon these particular studies (cf. Berrill and Liu, 1948).

The present account was completed before acquaintance was made with the work of Louis Agassiz of almost a century ago. Yet it was Agassiz (1862), with his profound belief in special creation and his idea of an organism as being as final 'as an expressed thought', who saw *Obelia* and other hydroids simply as they are. With the aid of A. J. Clark he described and pictured the hydroids in living detail that has not since been equalled. Our own account, even though independent, may well be regarded as a direct extension of Agassiz's observations, both in kind and in motive, being essentially a deep interest in and respect for the organism as such.

The Mature Colony

Obelia commissuralis McCrady is a species common along the New England coast, where it forms large branching colonies that attain a length of 20 cm. or more. Mature colonies consist usually of a few long stems bearing secondary and tertiary branches, and united proximally to form a narrow base of attachment. The extent and habit of growth sharply distinguishes it from *O. geniculata* and other species, *O. longissimus* being the only one with which it might be confused. The form of growth is related to the presence of long distal processes, with few branches and no hydranths, which are in fact large free terminal stolons. These are found at all seasons of the year and are not purely a response to high summer temperatures as in the case of *Bougainvillia superciliaris* (cf. Berrill, 1949). They give a distinctive appearance to the colony. The main lateral branches also end in stolons, though narrower and not so long.

Below the distal stolonic zone the hydranths are formed profusely. Gonangia develop only in the proximal regions of the colony, forming from the angles of hydranths and lateral branches. Annulations of the perisarc, usually three or four, separate the internodes and occur also at the base and tip of each hydranth stalk.

The questions presented by the general form and detail of a colony are the nature of stolons, wherever they appear, the development of hydranths, gonangia, and medusae, and the relationship between them; and the origin and significance of annulations, together with the formation and influence of chitin.

Growth Cycle

The preceding description presents a somewhat static picture. In reality there is a continual change in all parts of a colony, and the organism which is *Obelia* can be properly defined only as activity. This is sharply brought out by the behaviour of isolated pieces of distal stolon, especially when allowed to attach to glass at a temperature of about 15° C. Under these circumstances a cyclic transformation takes place.

The distal end continues to grow forward, though more slowly. The proximal end becomes progressively attenuated as cells degenerate and withdraw into the hydroplasm. The effect is a migration of the stolon piece as a whole, much like the motion of an amoeba in spite of multicellularity and slowness. No annulations appear in the attached stolon.

A short distance behind the advancing tip a vertical branch appears. This is annulated from the first, and at its distal end it develops into a hydranth. At the same time the stolen tip continues to advance and another and similar upgrowth arises a little behind the tip. The process goes on until such vertical branches with terminal hydranths stand more or less equidistantly down the whole length of the stolon. In the proximal half, however, there is not only a slow resorption of the basal attached stolon but resorption of formed hydranths.

The overall picture of the cycle is shown in Text-fig. 1c. Behind the advancing tip of such newly formed stolons, in orderly succession, there is usually a short annulated vertical branch, a longer growth with a developing terminal bud, one or more such branches with functional hydranths, a hydranth in process of resorption, and finally several perisarcal tubes containing coenosarc as attenuated as the basal stolon of this region. So it continues, new stolonic growth and new hydranths forming distally, resorption of the old occurring proximally, the resorbed material passing into the hydroplasmic stream and serving as nutrient for the building of the new.

PART I. STOLONIC GROWTH AND PERISARC

Stolonic Growth

Stolonic growth occurs at the free end of the main and lateral branches as part of the normal growth of this species. It also occurs to a more limited extent as basal attached stolons, and also from either end of cut pieces of stem. The process appears to be the same wherever it appears, except for variation in diameter, growth-rate, and the effect of contact.

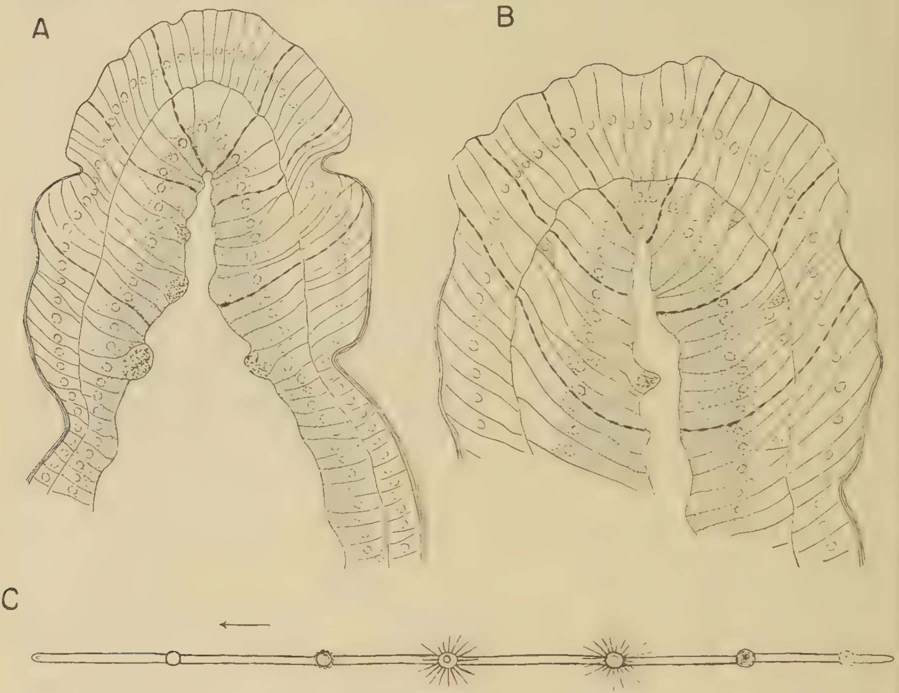
Rate of Growth

With optimum nutritive conditions, i.e. maintained either by actively feeding or by actively resorbing hydranths, the rate of stolonic growth varies directly with the temperature. Under such conditions the rate of growth at 10–12° C. is a little less than 1 mm. in 24 hours; at 16–17° C. it is about 10 mm. in 24 hours; and at 20° C. it may be as much as 15–20 mm. in 24 hours. Rapid growth continues at temperatures as high as 25° C., possibly 26° C., but at 27° C. growth ceases even though the tissue may appear to be otherwise healthy.

In most cases the maximum growth-rate possible for a given temperature is maintained only for limited periods, for production of developing hydranths behind the stolon tip introduces competitive demands upon the nutritive supply. Ideally, however, stolon formation is a process of rapid continuing growth with no recognizable decrement such as is characteristic of a developing hydranth or other organized structure.

PROCESS OF GROWTH

A detailed study of stolon tips reveals significant cell arrangements. Whether a stolon is massive or slender, the cells of both the epidermis and endodermis are streamlined as though flowing in and constituting a single current moving toward the tip. The intercellular contours of the endodermis continue with those of the epidermis. Whatever force is responsible for the configuration, the appearance is that the cells of both layers flow in conformity with the lines of force and are subordinate rather than determinate (Text-fig. 1A, B).



TEXT-FIG. 1. A, B. Sections through two stolon tips, respectively growing slowly and rapidly, showing confluence of cell contours of epidermis and endodermis (emphasized by heavy broken lines), and also showing graded vacuolation of distal endodermal cells. Note absence of vacuolation in the terminal cells of the more rapidly growing stolon (B). C. Diagram of growth sequence of isolated stolon, new stolonic tissue forming distally and old resorbing proximally, direction of growth indicated by arrow. In sequence along stolon are initial and late stage of developing hydranth, two mature hydranths, and hydrothecae of two resorbed hydranths.

Cell proliferation has been found only at the extreme tip of both epidermis and endodermis. In neither layer is there any sign of so-called interstitial cells and in any case they must be so rare that no significant role can be assigned to them. Since stolon growth is linear and in one direction, proliferation must in a sense be polarized, that is, the cells formed toward the proximal side of the proliferation zone cease division, those on the distal side continue to divide.

Immediately behind the proliferative tip the endodermal cells present a very distinctive appearance. Toward the hydrocoel or lumen they retain their characteristic granular nature, but on the side toward the epidermis the cells become extended with a large clear vacuole. Passing backwards from the tip, vacuolization rises rapidly to a maximum and then slowly decreases, finally disappearing altogether (Text-fig. 2B), when the cells become indistinguishable from those of the endodermis in general. When growth is very rapid the terminal proliferating endodermal cells may be granular throughout and somewhat smaller than the vacuolate cells. When growth is slower, the terminal cells themselves may be vacuolate.

The cell cycles appear to be as follows. In the terminal division centre of the endodermis the cells may be relatively small if division is very rapid, but if not so rapid, and in any case at the margin of the centre, the cells become greatly enlarged and vacuolated, the vacuolated cells probably dividing at least once. The vacuolation is probably a polar imbibition of water, is definitely a temporary phase, and is in some way related to the rejuvenescence or reconstitution of cell organization. The phenomenon is seen elsewhere in the activated epicardial tissue of the ascidian *Archidistoma* at the time of strobilation (Berrill, 1948).

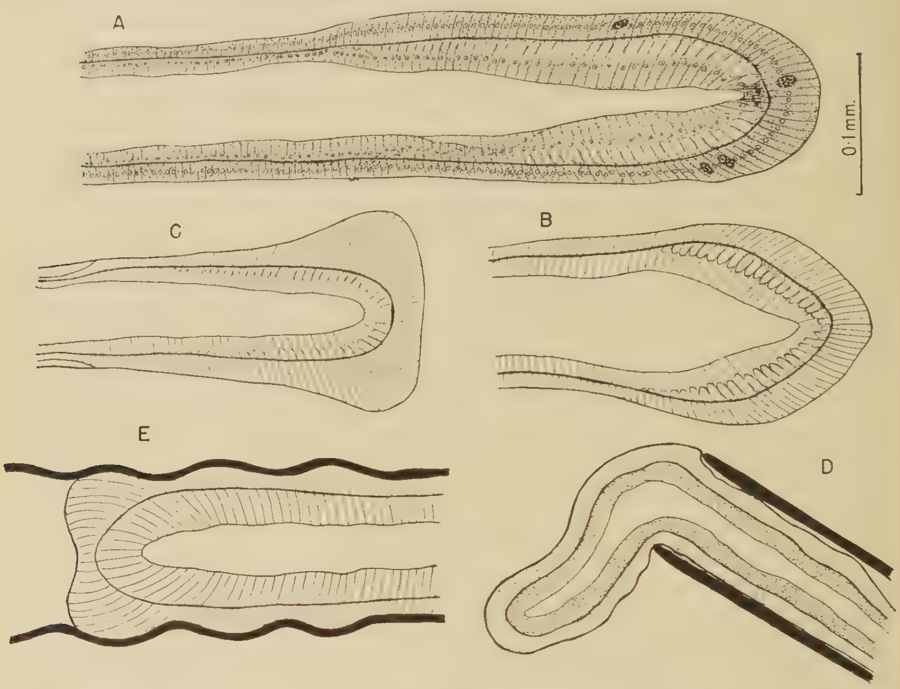
Whatever the significance of the polar vacuoles in terms of the physiology of dividing cells, they do afford a useful criterion of growth-rate. In slowly growing stolons relatively few new cells are produced in a given period, and correspondingly few cells are to be found in the vacuolated or recovery phase. In rapidly growing stolons, many more cells are formed within a given period and correspondingly more cells will be found in the vacuolated recovery phase. Each newly formed cell goes through its individual cycle in the same time. The extent and gradient in degree of vacuolation accordingly form an indicator of the biological or relative growth-rates, in contrast to actual growth-rates (Text-fig. 2A, B).

The epidermis exhibits a somewhat similar cycle. Cells in the proliferative zone are large and high columnar, and in this zone and in the recovery phase (assuming it to be comparable and coextensive with that of the endodermis) an extensive but less obvious vacuolated region exists external to the nuclei of the cells.

The flowing aspect of stolon tissue is shown even more strikingly when a piece is cut. When a stolon or branch is cut through there is an immediate closing over of the end of the endodermal layer to form a closed tube. The epidermis reacts in a similar manner but a little more slowly, as it flows up and around the tip of the endodermis. Both distal and proximal ends of a cut piece react in the same way. The response is so rapid that there is no possibility of cell proliferation being in any way involved. The condition

1–2 minutes after a cut was made is shown in Text-fig. 2E. The pseudo-fluid nature of the material is further shown by its behaviour under light glass pressure. The coenosarcal tube as a whole remains stationary and unaffected, but the tip slowly extends without significant change in shape.

When a cut is made across a stem or stolon some distance from its distal end, new growth occurs rapidly from the cut proximal end (Text-fig. 2D), that is, the normal polarity of growth may be reversed. When such outgrowths become attached to the substratum, growth may be vigorous and most of the old coenosarc and organized structures may resorb and become converted into nutrient for the new tissues.



Text-fig. 2. Growth of stolons. A. End of rapidly growing free stolon, showing thickened epidermis of the growing zone including a few glandular cells, and endodermis with a distal group of dense rapidly dividing cells grading into large vacuolated cells which decrease proximally in size and vacuolation. B. End of slowly growing free stolon, showing reduction in range but not extent of vacuolated zone and absence of non-vacuolated cells distally. C. End of slowly growing attached stolon, showing latero-distal spreading of epidermis. D. Stolonic growth from basal end of a cut stem. E. Distal healing in cut annulated zone of a stem, showing distal endodermal fusion and flowing of epidermis (cell division not involved).

Stolon tips readily attach to solid surfaces. Those already attached are difficult to detach without breaking them. Free stolons attach immediately upon contact, the region of attachment corresponding approximately with the extent of the recovery phase described above. In other words, the stolon surface is sticky for a limited though variable extent from the tip backwards.

When a free stolon attaches to glass, it assumes the terminal shape characteristic of creeping stolons in general. The contrast with the free stolon is shown in Text-fig. 2C. The endodermis remains unaffected, but the epidermis flows forwards and outwards as a pair of shoulders, that is, at the tip it has a tendency to spread on contact, clearly a surface tension effect.

HYDROPLASMIC STREAMING

Movement of the fluid within the hydrocoel has been generally noted. Huxley and de Beer (1923) recorded changes in contour of the coenosarc with the ebb and flow of the stream. Hammett (1943) reports the movement as streaming distally during hydranth development, proximally during hydranth resorption. Hauschka (1944) found from isolation experiments that the absence of the stream did not prevent hydranths from completing their development, and that the causal connexion suggested by Hammett did not hold.

While the present account is of *O. commissularis*, *O. geniculata* is essentially the same in this respect, and the obvious feature of the hydroplasmic streaming in *O. commissularis* is its regular reversal in direction. The rhythmic period varies with the temperature and may have from 3- to 7-minute cycle, consisting of a flow in one direction of from 1 to 3 minutes, a short period of quiescence, and a flow in the opposite direction of similar duration to the first. Cells, cell fragments, heavily pigmented cells derived from resorbed or resorbing hydranths all flow along with the stream.

The rhythmical streaming occurs in a stolon or piece of stolon in the absence of any formed or developing structures. It could be due either to a regular reversal in the direction of beat of the cilia lining the hydrocoel, or to contractions and expansions of one or both of the epidermis and endodermis. The activity of the cilia becomes most evident through the dancing movement imposed upon the travelling particles.

To determine whether cilia are in any way responsible for the current, let alone its reversal, various sections of stolons and stems were cut and studied in isolation. Only those that retained or recovered ciliary activity were employed. In no case could anything but local agitation be observed with no forward or backward current. There was no evidence that ciliary activity is responsible for the hydroplasmic streaming, and this is confirmed by observations on intact stolons in which marked ciliary activity is often seen to coincide with the absence of directional movement.

The alternative that coenosarcal contractions are responsible could mean either a local activity or a general peristaltic wave. Greatly enlarged superimposed series of camera lucida drawings showed no fluctuations in stolon diameter either in the recovery zone or down the main length of the stem, but in the region immediately proximal to the recovery zone a definite expansion and contraction was evident and coincided with the reversals of the stream (Text-fig. 3B, C).

The epidermis in this region when expanded comes into fairly close contact with the chitinous cuticle, when contracted becomes separated from it by a relatively wide space. The stream runs distally as the tube expands, proximally as it contracts. When expanded, ciliary activity and particle agitation and movement are at a maximum but when contracted no activity is discernible, due probably to sheer congestion of the solid particles. For the same reason the stream rarely penetrates the recovery zone, for cell enlargement in this region more or less occludes the lumen and leaves too narrow a channel for free movement of particles.

The rhythmical expansion and contraction of the coenosarc of this region could, however, be either passive or active, responding to pressure originating elsewhere or to the sucking up the hydroplasm by innate dilatation. That the streaming is due to and not the cause of the pulsation in the zone in question is shown by the following observations.

If stolon tips, including the pulsation zone, are cut off while the stream is flowing distally, it stops immediately in the part remaining. If it was being pushed along rather than pulled, the stream should continue to flow through the cut end, which it does not. When flowing along a stem which forks, the stream should enter both branches if propelled from below. It does not and will by-pass wide open channels to flow along a single passage, which is in conformity with a distal pull.

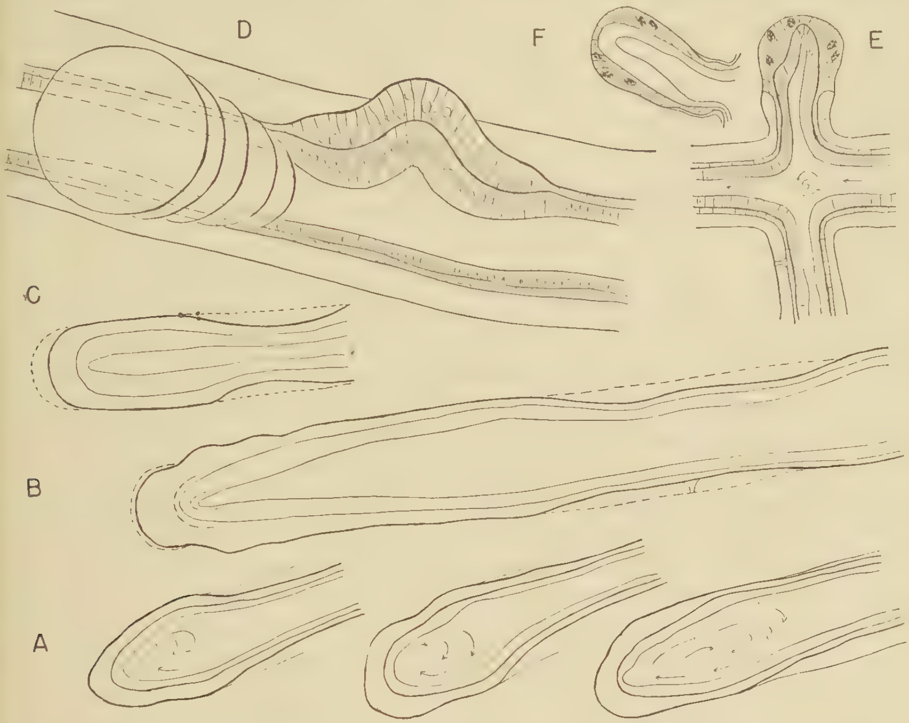
Both epidermis and endodermis are involved in the pulsation and adhere closely together. It is not obvious whether both are responsible or only one. Slight changes in thickness of the epidermis at first suggested that this layer was the contractile tissue, but other evidence indicates that it is either endodermis or the junction between the two layers that contracts and expands.

Where recovery and pulsating zones are short, and the transition gradient steep, the relative role of the two layers is more clearly seen, as in attached slowly growing lateral stolons (Text-fig. 3E, F). When expanded, the epidermis comes into contact with the chitinous perisarc. When contracted, the epidermis gives the appearance of being pulled away from its adhesion, leaving several but varying cells attached to the perisarc on each occasion. The appearance suggests that the epidermis is passively pulled between two surfaces to which it tends to adhere.

Myofibrillae have not been detected histologically, and the rate of contraction and expansion is much slower than would be expected even in the most sluggish fibrillar system. It has more in common with the contractile epithelium reported for ascidians (Berrill, 1929) and the cortical movements of teleost eggs and blastoderms (Yamomoto, 1940).

The extent of the contractile zone is variable and is much the same and in proportion to the extent of the recovery zone. In a progressively growing stolon, the contractile phase is of limited duration for any individual cell, but the faster new cells are produced the greater will be the number that will be in a given phase, whether recovery or contractile, at a particular moment. When the contractile phase is passed, the cells become quiescent endodermis, ageing slowly with time, although any stimulus that produces local cell division evokes the whole cycle anew.

There is some indication that the vacuolation–contraction cycle is less a response to a recent cell division than it is to cell growth as such, division of the cell being itself a response to the same condition. This is suggested by the appearance of the large cells initiating the outgrowth of a lateral branch (Text-fig. 3D) and by the short period (2 hours) that elapses between the cutting of a stem and the presence of vacuolate and contractile regions.



Text-fig. 3. Stolonic pulsation. A. Camera-lucida drawings of same stolonic tip 2 minutes apart, showing changes in diameter and contour and varying extent of internal ciliary activity. B. Rapidly growing stolon, with relatively large terminal flow of epidermis; broken lines represent contour alternating with that of unbroken lines, showing slight terminal pulsation, and extensive pulsation zone proximal to thick vacuolated zone. C. Similar to B, showing two particles adhering to epidermis and exhibiting merely transverse movement with the pulsation. D. Lateral outgrowth starting from junction of stolon and base of hydranth stem; both epidermal and endodermal cells greatly enlarged as preliminary and accompaniment of proliferation. E, F. Contracted and expanded phases of a pulsation of a lateral attached stolon, showing tendency of epidermal cells to adhere to cuticle during contraction phase.

Branching

Branching occurs both from creeping stolons and from erect stems and free stolons.

Creeping stolons. Vertical branches are the first to appear, from the upper side of a stolon a short distance behind the tip. The region from which they arise, one at a time, at *the time of origin*, is the proximal part of the contractile or pulsating zone. As the first branch grows vertically, the stolon tip has usually undergone further growth of its own, the pulsating zone following close behind the tip and leaving the vertical growing branch attached to a region that loses its pulsating property. As the stolon tip continues to grow, the distance increases between it and the base of the branch, the pulsating zone migrates forwards to a corresponding extent, and after the first branch has been left behind a certain distance, a new vertical branch forms from the upper side of the pulsating zone. This process continues more or less indefinitely (Text-fig. 1C).

The vertical branches thus formed are at first always annulated, whatever may be their ultimate fate. After several such branches have formed, a tertiary outgrowth of a stolonic nature appears close to the junction of a vertical branch and the main stolon, the youngest junction to exhibit tertiary growth usually being the third from the tip, more rarely the second or fourth (Text-fig. 3D). In other words, it forms at a junction after that junction has existed for a certain duration of biological time.

This last type of outgrowth is always lateral and in contact with the substratum, growing out at right angles to the primary stolon. In a comparable manner, after it itself has existed for a certain period, another lateral outgrowth appears opposite it in the same plane, also in contact with the substratum. Neither group of contact stolons ever exhibits annulations.

The localization of outgrowths in the two regions just described, i.e. the pulsation zone and the branch-stolon junction, have features in common which distinguish them from other parts, namely, a wider lumen, a current system that affords greater opportunity for intracellular feeding, and probably better metabolic conditions generally as the result of more space and effective ciliary activity. If ciliary activity can be taken as a fair index of respiratory conditions, there is little doubt that such conditions are better in these two places than elsewhere, in the one due to the innate pulsations, in the other to the hydroplasmic junction pressure created by the pulsating branch terminal.

In the case of free stolons or stem terminals, the pattern of outgrowths is essentially the same as in creeping stolons. The difference here is that all branches are at first annulated, again indicating that contact with a solid substratum effectively suppresses any tendency to annulation.

In conclusion, there appears to be but one stolonic activity in terms of cell cycle and branch formation, modified only by growth-rate and contacts.

PERISARC

Free stolons may or may not exhibit annulation. Whatever its extent, it cannot properly be discussed except in terms of the chitinous perisarc which retains the annulation even after the molding tissue has lost it.

Little study has been made of this substance and its role in the economy of hydroids. There are some unexpected properties. Weismann (1883) included an illustration, obviously accurately presented, that Nutting (1915) doubted on *a priori* grounds, showing an outgrowth rudiment secreting its own fine perisarc and protruding through a hole in the thick chitin surrounding an old stem. Louis Agassiz (1862) in fact illustrated almost exactly the same thing for *O. commissularis.* Taking Weismann's illustration at face value, the chitinous perisarc clearly can both be laid down and dissolved under certain more or less obscure conditions.

Hammett calls attention to the elasticity of the perisarc at the distal end of nearly developed hydranths and speaks in terms, no doubt correctly, of polymerization of the secreted substance. The brittle rigidity of old perisarc in contrast to the sticky stretchiness of the newly formed material of stolons and developing hydranths suggests forcibly that polymerization does take place.

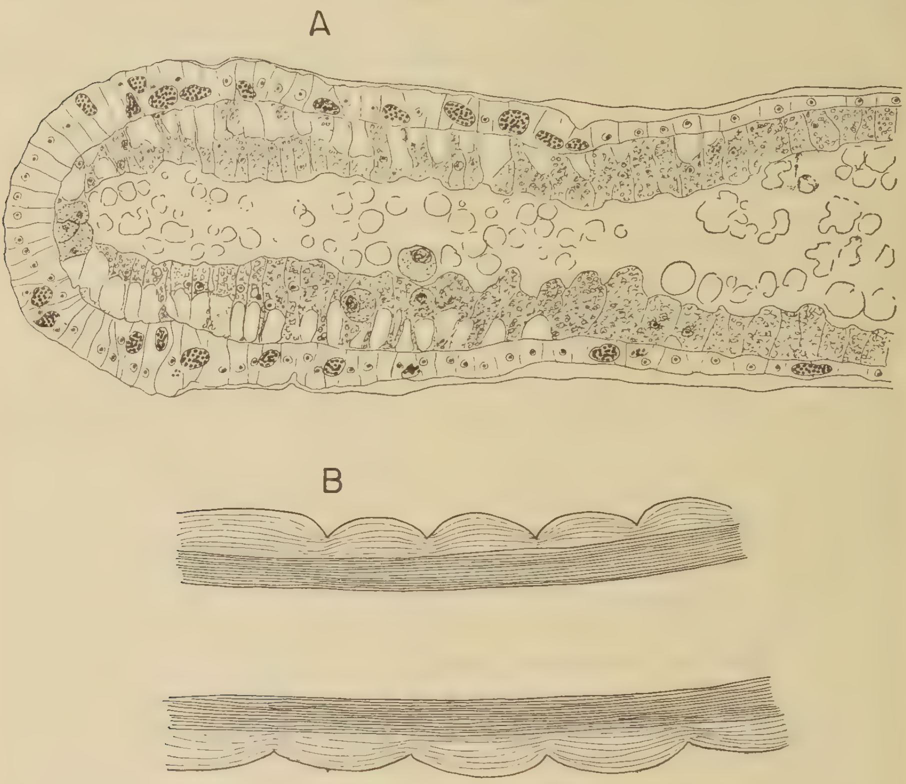
The increasing thickness of the perisarc with the age of a stem, its laminar quality, and above all, the formation of straight lamellae on the inner side of annulations, demonstrates that chitin production is continuous and not limited merely to growing regions (Text-fig. 4B).

Perisarc is secreted at the growing end of a stolon, though hardly visible in the recovery zone, yet sufficiently for a very thin though non-elastic film to be present at the distal edge of the pulsation zone, gradually thickening proximally. In the pulsation zone it is no longer adhesive (unless previously attached) and is probably fully polymerized. If polymerization is rapid, as it appears to be, the various shapes permanently assumed by the chitin in conformity with the shape of the secreting tissue is explained. Straight stolons produce straight cylinders, curved surfaces produce curved perisarc.

The evidence is that chitin is secreted continuously and not only by growing regions. It is therefore either a general property of epidermal cells or else specialized cells must be present. If the former, then the property is apparently lost in the case of further specialization, as in tentacle epidermis. There is evidence, however, that specialized cells are solely responsible, and that the greater part of the epidermis is basically unspecialized.

Distinctive ovoid cells are always to be found in the epidermis of a growing stolon tip, and in somewhat smaller numbers throughout the rest of the epidermis. Similar cells occur throughout the stem epidermis, lying parallel to the surface. They are not typical epidermal cells and undoubtedly have some special function. They may well be the cells Weismann concluded were rudimentary ova, which would then need to migrate to other sites, and led him so far astray in his interpretation of hydroid reproduction. Being larger, rounder, and more granular than other cells of the epidermis, they might be interpreted as rudimentary ova if the desire to do so was excessive.

Their most characteristic feature, however, is that the cytoplasm is packed with highly refringent and insoluble granules, as clearly visible and refringent in the living cell as in preserved material (Text-fig. 4A). In the cells when alive and when preserved in formalin, though not stained, the contained granules have the same appearance and optical properties as the chitinous cuticle, and it is difficult to avoid the conclusion that these are glandular cells responsible for the secretion of chitin. Chemical tests for chitin are crude, however, and in any case there is no guarantee that the staining properties of secreted chitin and of its mother substance would be the same. While iron haematoxylin has no special affinity for chitin as such, it does stain the intracellular granules as deeply as chromatin, and darker than the inclusions of any other cells (Text-fig. 4A).



Text-fig. 4. A. Section of free stolon, stained with iron haematoxylin, showing numerous glandular cells with deeply staining granules in epidermis, and the three endodermal zones consisting of distal non-vacuolated cells, vacuolated zone, and proximal non-vacuolated zone. B. Chitinous perisarc of old stem, showing external loosely laid lamellae laid down during annular growth, and closely packed straight lamellae laid down subsequently.

On other grounds entirely, it is plausible that these cells secrete chitin. They must have a function, they appear to be secretory, are epidermal, and are adjacent to the only epidermal secretion recognizable. Their distribution in *Obelia* and other hydroids is such as to account well for the presence and nature of perisarcal structures. They also make possible a dual control mechanism for perisarc that would be difficult to account for otherwise. Special cells lay down the chitin in successive layers, while ordinary epidermal cells when dividing or undergoing rapid growth are able to dissolve it.

ANNULATIONS

The annulations of the perisarc of hydroids have long been regarded as diagnostic features of specific importance. Hyman (1940) suggests that they add strength to the stems and branches of a colony, but this is unlikely since stems more often break at an annulation than anywhere else. As formed structures they apparently have little importance to the organism, but as records of past activity of growing points they are invaluable. They are products of epidermal fluidity and chitin polymerization.

A typical annulating stolon is shown in Text-fig. 5C, D. As in simple stolons, the flow lines of the epidermis and endodermis coincide, but there is a striking difference. The epidermis, flowing distally as it grows, extends far beyond the endodermis, to such an extent that it tends to form a spherical blob or drop (Text-fig. 5E). With further growth, the tip of the endodermis enters the blob and may reduce its basal curvature (Text-fig. 5F). In the stolon shown in Text-fig. 5E the endodermal tip has passed through and widened the penultimate blob, while the epidermis has grown beyond and formed one more.

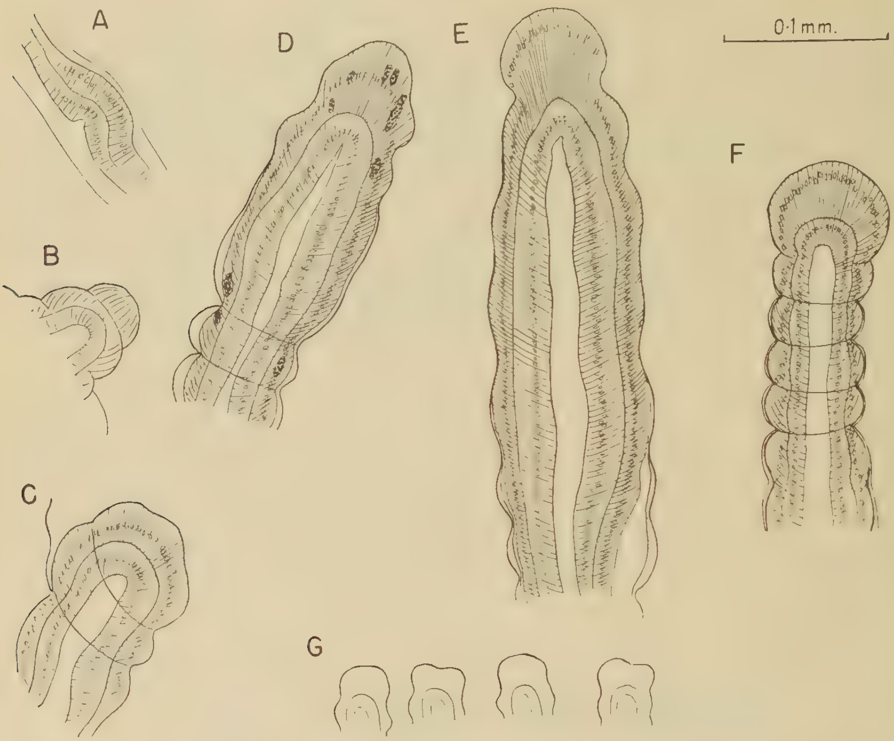
The lagging behind of the endodermis is not necessarily due to a slower rate of growth, but is at least in part the result of a slower start. The initial rudiment (Text-fig. 5A) bulges out and only after the epidermis has formed a hemispherical mass does the endodermis gather enough substance to follow (Text-fig. 5B). That is, there is an initial epidermal head start, expressed as a single annulation, unless the epidermis is in contact with the substratum, in which case the two-dimensional spreading interferes.

If the growth of the endodermis forces the pace a little, it catches up with the epidermis and the two tissues grow onward together as a simple stolon with a few basal annuli. Under certain conditions, such as starvation at moderate temperatures, initial growth-rates are maintained and attenuated stolons annulated throughout their length are frequently seen.

The formation of distal epidermal blobs entered from behind by the endodermal terminal presents little difficulty, but there is the additional feature to explain, that the epidermal annuli persist for a while and the perisarcal annuli permanently, after the endodermal tube has passed through. To resist the flattening influence of the internal pressure and maintain superficial curvatures, a degree of surface solidification is essential.

Chitin when first secreted at the surface flows to form a barely detectable viscous film conforming to the shape of the epidermal blob. At the extreme tip where all cells are probably in process of division it is virtually non-existent. The film forms more definitely on the sloping sides, and solidification or polymerization of the film over the proximal part of the blob occurs before the endodermal tube enters it. At temperatures between 15° and 20° C. it has about one hour in which to polymerize, this being approximately the time a blob takes to form before being entered by the endodermis. If the growth-rate is relatively fast and the available time at a minimum, some flattening is produced by the endodermal tube and annulations become shallow (Text-fig. 5E); when slow, the constrictions remain deep (Text-fig. 5F).

The succession of epidermal blobs may represent rhythmical growth surges, although it is not necessary to postulate this since a significant analogy may be made between the continuous flowing of water though a small opening and its subsequent subdivision into drops by the action of surface tension forces.



Text-fig. 5. Annulated growth. A. Initiation of outgrowth, enlargement and division of cells of coenosarc wall. B. Early stage forming second annulus, epidermis forms blob in advance of endodermal growth. C. Two-annulus stage, with blob entered by endodermis. D. Later stage succeeding first phase of annular growth, nearing end of stolonic phase, with epidermal blob again forming (glandular cells also shown). E. Similar stage with fully formed epidermal blob and lagging endodermis. F. Final blob at end of second sequence of annular growth, forming hydranth rudiment. G. Terminal contours of epidermal blob drawn 1 minute apart.

With progressive growth of the coenosarc as a whole, the region in which certain annuli are formed in effect travels down the stem, while the coenosarc passes into and out of its pulsation phase and withdraws from its contact with the perisarc. The chitinous perisarcal annuli in consequence enclose a straightened coenosarc. Chitin secretion continues, however, and the chitin is subsequently deposited as straight lamellae on the inner side of an annulated outer layer (Text-fig. 4B).

The mobile fluid-like nature of the epidermal blob is illustrated in Text-fig. 5G. In contrast to the fixed contour of the base of the blob, the distal part is continually changing every few seconds from a multiple to a single curved surface and back again, the single curvature predominating.

An analogy of some significance in the light of the following sections on morphogenesis, is that of a glass blowers' terminal blob of molten glass (epidermis), with a column of air (endodermis) being blown into it through a tube.

PART II. HYDRANTH DEVELOPMENT

There is a sequence of events leading to the initiation of hydranth development which in *O. commissularis* has so far been found to be invariable.

The hydranth always arises from the end of a short stolon that has undergone the following sequential changes, namely, several initial annulations, a phase of simple (non-annulated) stolonic growth, followed by a second and distal series of annulations.

The first series of annulations is due mainly to the head start of the epidermis. This overhead, however, is progressively lost as the endoderm in effect catches up, and as growth-rates and terminals coincide, straight stolonic growth becomes established. After a variable period of such growth, the growth-rate of the endodermis begins to fall off again relative to that of the epidermis, and terminal epidermal blobs (and annuli) again appear.

There is a difference in the annulated growth of the first and second series. In the first it is a diminuendo, the epidermal blob becoming progressively reduced in size at the time the endoderm penetrates it, until none is formed. In the second it is a crescendo, the endodermis lagging more and more so that the terminal epidermal blob is larger each time it is formed. With the third, fourth, or fifth epidermal surge, a crisis is reached.

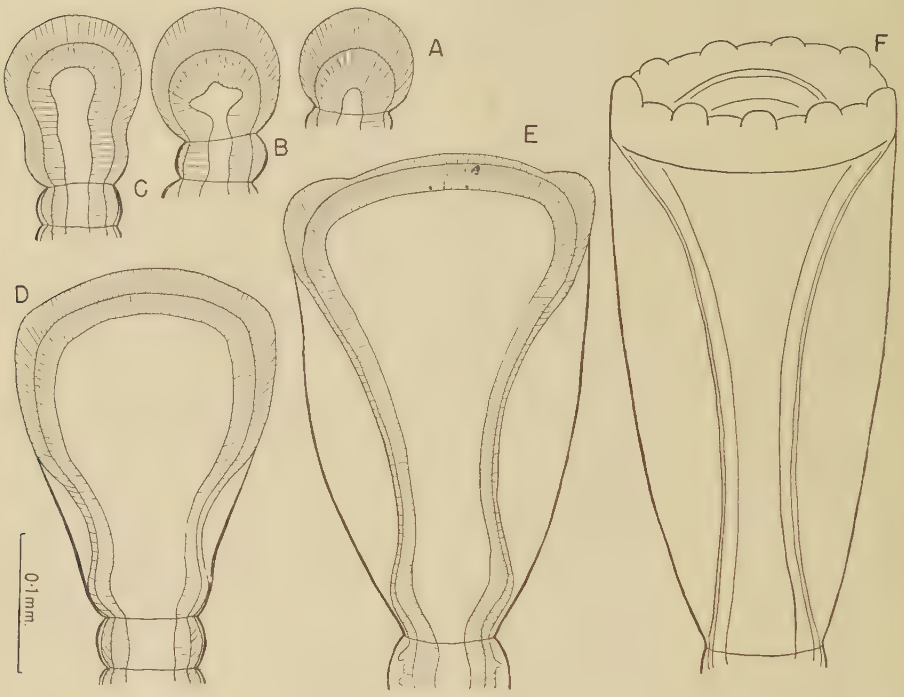
The sequence just described, culminating in the cessation of the second series of annulations, is characteristic of a relatively low temperature range, below 18°–20° C. Above this level, in most circumstances, the phase of simple stolonic growth succeeding the first series of annulations continues indefinitely. The appearance is that the endodermis responds to higher temperatures a little more vigorously than the epidermis, becomes the pacemaker and enforces simple growth.

At relatively low temperatures, the absolute reduction in growth-rate of both tissues, and the relative lagging of the endodermis result finally in the formation of an epidermal blob larger than its predecessors, and entered by the endodermis even more slowly than before. Both features are important, the greater quantity of material in the unit, and the longer time in which something can happen.

The terminal unit shown in Text-fig. 5F is typical of the new starting-point, with maximum epidermal blob enveloping the tip of the endodermis.

This is the rudiment of the hydranth. The unit now grows as a whole and quantitatively exhibits a typical growth-curve or decrement until all growth ceases.

The slowing of the growth-rate of the endodermis is indicated by the disappearance of the terminal zone of relatively small rapidly dividing cells,



TEXT-FIG. 6. Hydranth development (chitinous cuticle in heavy black). A–E. Note changing range of vacuolated zone. A. Initial semi-hemispherical rudiment with terminal epidermal blob fully entered by endodermis. B, C. Enlargement and elongation as cells become added from the anterior disk to the proximal cylinder. D, E. Progressive growth, limitation of vacuolated cells to anterior disk, proximal formation of pulsation zone causing detachment of coenosarc from the perisarc. F. Slightly later stage at same scale showing complete separation of coenosarc and perisarc, and conversion of marginal rim of disk into rudiments of primary and secondary tentacles.

and the substitution of dividing cells of maximum size and vacuolation. The effect is to give expansion a lateral as well as a distal direction, resulting in the slow formation of a flask-shaped structure (Text-fig. 6A, B, C).

At the same time, apart from change in diameter, which increases at the distal end, and in the absence of a centre of maximum growth-rate, the growth sequence of cells is basically similar to that in stolon growth. Cell division continues in the area extending from the distal centre to the shoulder slope, vacuolation of the endodermis being at a maximum in this region, the epidermis being comparable in its own way. Behind the shoulder, tapering toward the neck, vacuolation decreases and disappears near the junction with the annulated stalk (Text-fig. 6C).

As growth proceeds, the distal disk of cells becomes wider. More cells pass from the edge of the disk into the distal part of the slope. Vacuolation disappears in cells farther and farther from the junction with the stem. Epidermal cells become progressively less columnar in company with those of the endodermis.

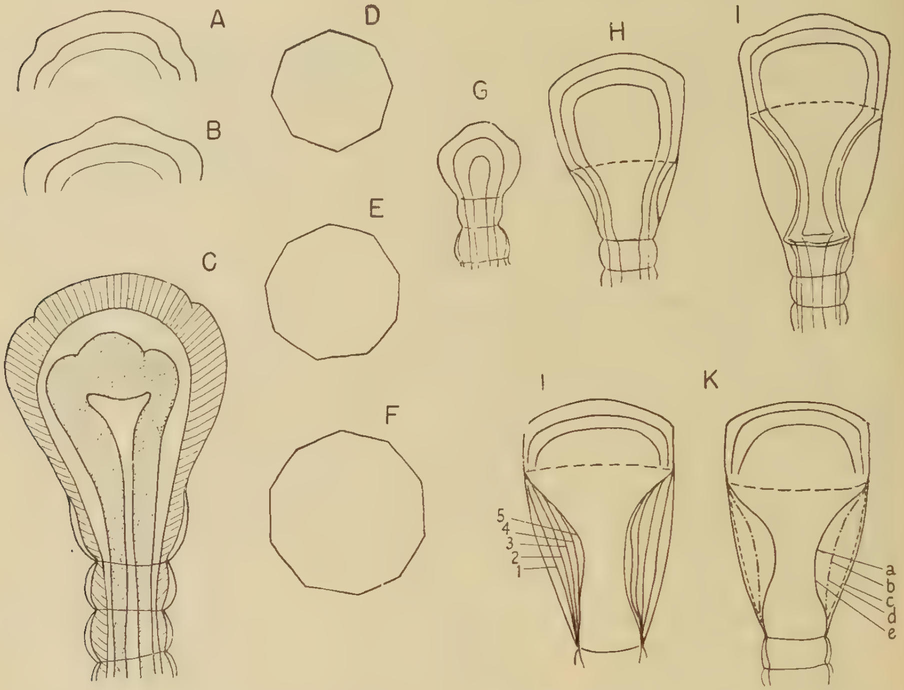
With the proximal disappearance of vacuolation, a zone appears that corresponds in relative location to the pulsation zone of the stolon, and with its appearance the pulsations commence. At first the extent and the amplitude are such as to be barely detectable. With progressive distal addition to the area, both the extent and the amplitude increase, the rhythmical movements become obvious, and the epidermis is pulled away from the chitinous perisarc (Text-fig. 6D, E).

The pulsations in the hydranth have been reported already for later stages in *O. geniculata* by Hammett (1943), who regards them as the result of hydroplasmic ebb and flow originating elsewhere in the colony. To test this in *O. commissularis*, various stages of developing hydranths were isolated by cutting through the stem in the distal annulated zone. The reversing hydroplasmic stream in the intact stems ceased immediately, while the rhythmical contractions and expansions in the hydranth buds continued indefinitely. Accordingly there is no reason to doubt the existence of active pulsation in the hydranth itself, while the manner of formation of the zone and the identity of rhythm rates leave little doubt that the phenomenon in hydranth and stolon tip is the same.

The pulsation zone is gradually extended distally until the bud consists of a long pulsating cylinder of flat and cuboidal cells, surmounted by a cap of columnar epidermal and endodermal cells that forms a right angle junction with the cylinder. The cylinder becomes the stomach of the hydranth, and the pulsations persist almost until the hydranth as a whole becomes a functional entity. The extent of growth of the whole from the bud rudiment to the present stage is shown to scale in Text-fig. 6A–F. Text-fig. 7J shows five consecutive positions of the cylinder wall in process of contraction, Text-fig. 7K five positions occupied one minute apart.

As the round bottom of the inverted flask (Text-fig. 8A) becomes converted into a flat bottom, a new crisis is reached. No further cells are added to the slopes to augment the pulsation zone, while other events take place at the marginal junction. The profile of this stage is shown in Text-fig. 8B. The cylinder is surmounted by an extremely straight-sided ridge or flange, which in turn surrounds a slightly convex disk. Both ridge and disk, in contrast to the pulsation zone, consist of high columnar cells in progress of slow division. The inner surface of the endodermis of this region bears very active cilia.

The interest in this stage concerns the marginal rim. Seen in lateral profile, nothing appears remarkable, but examined from the more difficult angle of above or below, the circumference is seen to be no longer circular but many-sided. The number of sides varies from 8 to 12, according to the size of the hydranth bud at this stage. Imperceptibly, the circular outline of a slightly earlier stage flattens equidistantly to form 8, 9, 10, 11, or 12 sides (Text-fig. 7D–E), the length of a side being approximately the same in all, and the number of sides being directly proportional to the length of the circumference. This foreshadows tentacle formation, and the number of tentacles that will be formed is twice the number of sides, whatever that may be.



Text-fig. 7. Hydranth development. A, B. Alternating contours of distal end shortly before tentacle initiation. C. Similar stage with endodermis shrunken as consequence of narcotization, showing marginal growth of epidermis and endodermis independently. D, E, F. Distal end-views of stage I and Text-fig. 6E, showing polygonal form, and variation in number of sides with length of circumference. G, H, I. Three stages of early development drawn to same scale as in Text-figs. 8 and 9. J. Pre-tentacle stage showing five consecutive contours of cylinder wall during contraction. K. Same stage, showing position of cylinder wall during expansion and contraction 1½ minutes apart.

At 20° C. it takes about 4 hours to pass from the stage where polygonal structure is suspected to the definite configuration, a relatively long period in view of the rapidity of subsequent changes.

As the cylinder alternately expands and contracts, the contour of the distal region alternates between those shown in Text-fig. 7A and B. At one time the marginal thickening appears to be epidermal, at the other it seems to be endodermal. It is difficult to determine which of the two layers is really responsible for the variable thickness of the whole, even when vitally stained with brilliant cresyl blue or with neutral red. However, a prepared slide was discovered representing stained specimens that had clearly been narcotized preparatory to fixation, and while formed structures and the epidermis throughout appeared to be in excellent condition, the endodermis of rapidly growing regions was shrunken or withdrawn. In one instance, at the stage in question, a space had appeared between the two layers (Text-fig. 7 c), and it is evident that in both epidermis and endodermis growth has resulted in the differentiation of a thick marginal rim and a central dome. Both layers are involved.

After the distal polygon has been definitely established, a relatively, even remarkably, rapid transformation occurs. Each epidermal corner becomes rounded, again like a bead induced by surface-tension. Only a few minutes later the unorganized territory remaining between adjacent corners also draws up into small hemispherical masses (Text-fig. 6F, 8C).

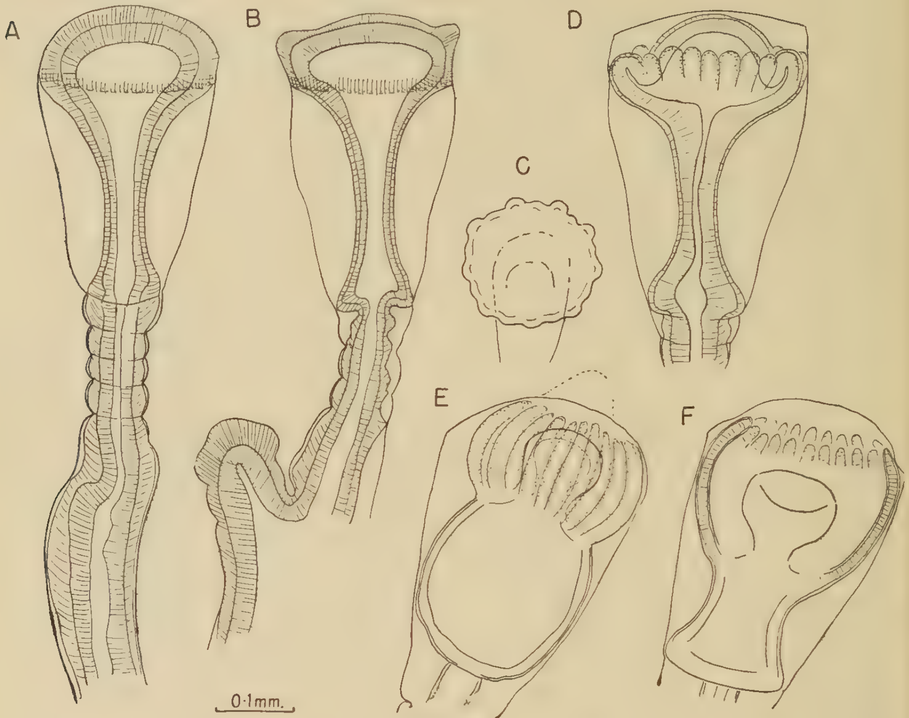
The row of beads thus formed at the margin of the disk represents the full number of tentacles that are to be formed, those arising from the corners giving rise to the more or less horizontal tentacles of the expanded hydranth, the intermediate ones becoming more or less erect.

The tentacles of *Obelia* are small and as they are initiated on a correspondingly small scale, they are less suitable than those of many other hydroids for an analysis of tentacle histogenesis. At the same time, a tentative analysis is as follows. Local proliferation of marginal endoderm results in the delamination of masses of very small cells, a similar process occurring in the adjacent epidermis. The small endodermal cells enlarge individually, and constitute the endodermal chordal cells of the tentacles without further increase in number. As the epidermal covering becomes thrust out, the small cells of epidermal origin are also drawn out, giving rise to the nematocysts.

The final phase of hydranth development occupies but a few hours, and consists mainly of differentiation processes in the three zones already established (Text-fig. 8D). The central dome grows into a thin-walled manubrium, opening distally to form the mouth. The marginal beads rapidly grow out as the tentacles, while the stomach forming from the wall of the cylinder acquires typically pigmented gastrodermal cells, with inclusions that stain intensely with neutral red.

Simultaneously, movements commence indicative of the functional differentiation of muscle-fibres. Twitch contractions appear in the tentacles, occupying merely a fraction of a second. In the stomach, movements occur which change the shape rather frequently, each transformation occupying but a few seconds (Text-fig. 8E, F). Similar movements develop in the manubrium though somewhat later. While these enteric contractions are slow compared with a tentacle twitch, they are very fast compared with the rhythmical pulsations described earlier, and there is no possibility of confusion between them.

Hammett (1943) has recorded for *O. geniculata* the necessity of rhythmic surging of the whole hydranth for the final stretching and rupture of the thin sticky perisarc covering its distal end. In *O. commissularis* it is not certain that a coat of quite such consistency is present. In many cases the hydrotheca retains its polygonal character, though it is slight and often disappears.

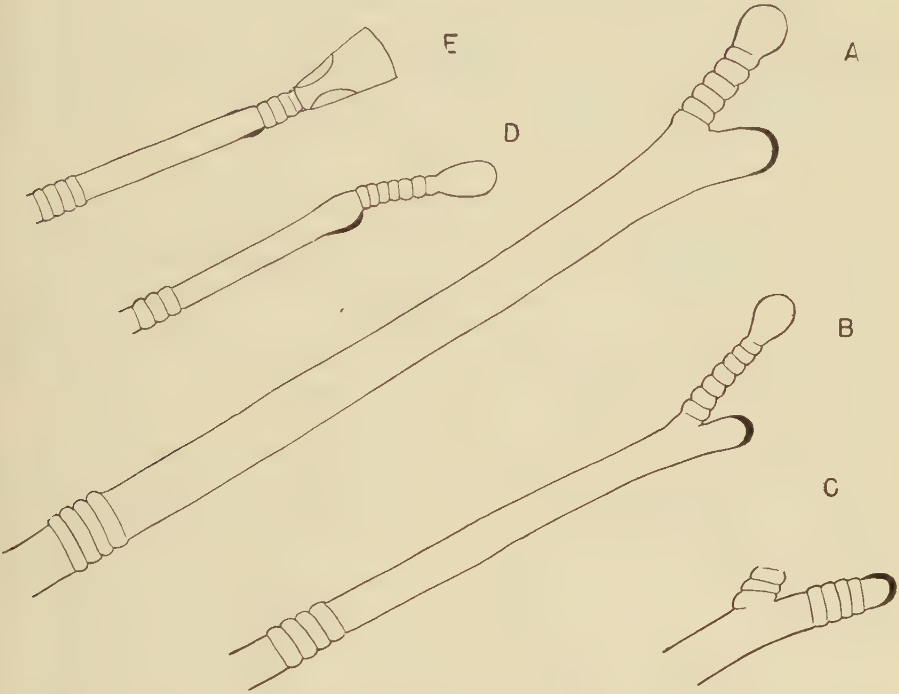


TEXT-FIG. 8. Later development of Hydranth. A, B. Late pre-tentacle stages, showing origin of new terminal growth from wall of distal part of non-annulated or internodal stem. C. Distal view of initiation of primary and secondary tentacles. D. Lateral view of stage immediately following that of Text-fig. 6F. E, F. Later stages with dilated and contracted body-wall, showing elongating tentacles and formation of manubrium.

With the opening of the mouth, the large pigmented gastric cells derived from the resorption of hydranths elsewhere in the colony and accumulated in the cul-de-sac of the developing hydranth, escape to the exterior. In young hydranths they may be seen as a ball of yellow-orange material, opaque by transmitted light, held between the tentacles.

It was mentioned earlier that hydranths usually do not start to form unless the temperature is below 20° C. If after development has started the temperature rises even several degrees above this threshold, development continues to completion. At temperatures in the region of 25° C., however, the hydranth reaches the functional state and then usually resorbs immediately, indicating a greater susceptibility of fully differentiated specialized cells to high temperatures than either cells in the developing system or in the unorganized coenosarc. The process of hydranth resorption has been adequately described by Huxley and De Beer (1923).

One phenomenon remains, of some interest in *O. commissularis* itself and of considerable interest when comparisons are made with other species and genera. This is the manner of terminal growth of main and subordinate branches. The main stems of a colony and the stems of the next order are similar except in diameter (Text-fig. 9A, B). In these two cases, when the second annulation series is complete and the hydranth bud has reached the flask-shaped stage, new stolonic growth has emerged from one side of the anterior internodal stolonic region. This in turn undergoes its first annulation series and then converts to stolonic growth (Text-fig. 9C).



TEXT-FIG. 9. Terminal branches of colony, all to same scale. Heavy crescent represents distal growing zone. A. Terminal of a main stem, B, of secondary branch, showing similarity of growth form and difference in stem diameters. C. Continued growth of distal end showing primary annulation phase and commencement of internodal simple growth. D. End of branch of 3rd and 4th order, showing belated initiation of terminal growth. E. Remote lateral branch terminal with developed hydranth and suggestion only of new growth zone.

In terminals of branches of the third or fourth order, a difference appears. When the hydranth bud has reached a comparable stage (Text-fig. 9D) the new terminal growth arising from the distal non-annulated region has barely started, while in branches even more remote from the main stems hydranth development may be complete before the new growth starts (Text-fig. 9E), and in fact may arrive at a condition where there is a functional terminal hydranth and no further growth at all.

PART III. GONANGIUM AND MEDUSAE

The production of gonangia raises the following questions. Where and when do they arise, and why? What are the formative processes initiating their appearance? How does the early developmental phase differ from that of a hydranth, what are the developmental consequences, and what other factors intervene to bring about further differences?

Place of Origin

In this species the gonangia arise only in the basal and central part of a colony, that is, from the older parts. They arise generally from established junctions, regions where a stem junction already exists. From such places gonangia are second outgrowths, although second outgrowths need not be gonangia. In the same general region of a colony, though always toward the proximal end of a branch, gonangia may arise from the unbranched internodal pedicel of a hydranth.

Since junction zones tend to become larger with age, the diameter of such a zone might relate to the size of outgrowths emerging from it, and this in turn be a determining factor. On the other hand, those arising from simple hydranth pedicels would remain unaccounted for, and in any case the initial gonangial protrusions do not appear to differ from those leading to hydranth development. It is therefore more likely that the primary determinant is metabolic rather than physical or dimensional. If the lower growth-rate of lateral branch terminals is taken as a criterion of metabolic conditions, it indicates a lower growth tendency in the basal parts compared with the apical parts of a colony. The rates of stolonic outgrowth from pieces of stem isolated from various regions confirm this.

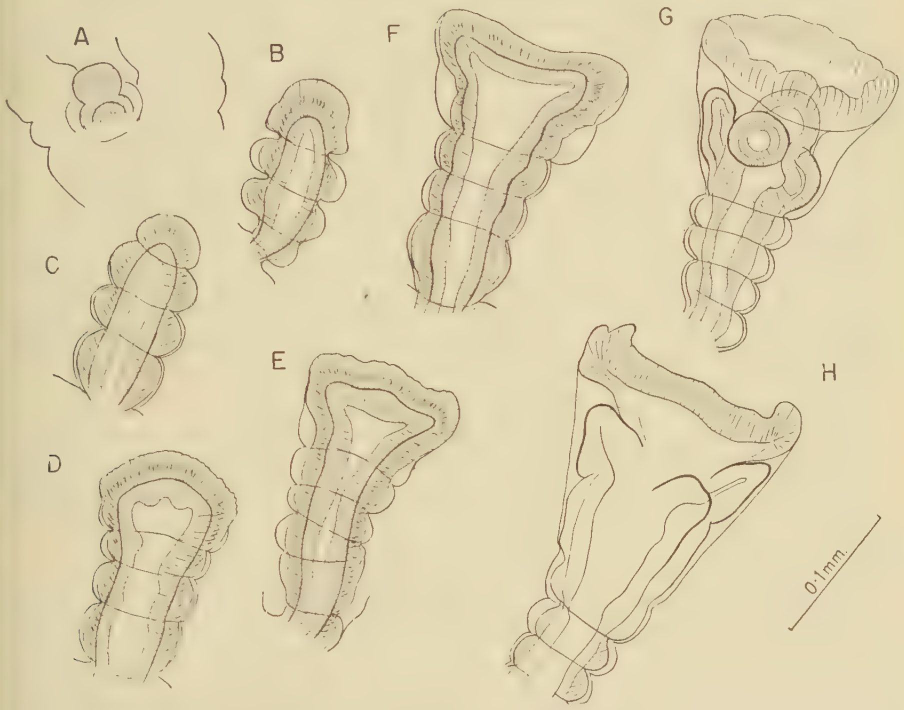
Growth Sequence

While the initial protrusions appear to be similar for hydranth and gonangium, the succeeding growth sequences are very different. The hydranth sequence consisted of a series of annulations of diminishing diameter, changing to simple stolonic growth culminating in annulations increasing to form the hydranth rudiment.

In the gonangial sequence, annulating growth commences as before, but the endodermis not only fails to overtake the epidermal blob, but lags so that the terminal cap enlarges with each annulation. Three or four annulations occur in this manner, corresponding in kind to the second series of the hydranth stalk, but forming here as the primary series (Text-fig. 10A–E). Sometimes the number of annulations may be much greater, but it is still a crescendo and the final state is the same.

GONANGIAL RUDIMENT

As the final surge occurs, which produces the gonangial rudiment, another difference appears. The final unit is flatter and wider than the hydranth rudiment, and from the first consists of a considerably larger number of cells (cf. Text-fig. 6A and 10D). This difference was noted by Weismann (1883) and by Hammett (1943), who compares the two primary caps with a cylinder (hydranth) and tam-o'-shanter (gonangium). However, this is a difference that develops rather than one which at first exists.



TEXT-FIG. 10. Development of Gonangium. A. Initial stage. B, C, D. Primary annulation growth phase with expanding diameter culminating in gonangial rudiment. E, F. Growth of rudiment, especially of disk. G, H. Initiation of medusa buds from wall of anterior cylinder, and consequent shrinkage of distal endodermis (stippled in G).

If we compare the formative blobs at the time of endodermal entrance, the difference is less striking. It is none the less significant. The distal flow of the epidermis is reduced and there is greater lateral spreading, so that more cells lie in the transverse plane, and the greater total number of cells present in the initial gonangial rudiment both lead to the development of a larger final structure, final size being a reflection of initial scale of organization.

DEVELOPMENT OF THE GONANGIUM

The unit which continues to enlarge as the developing gonangium shortly consists of a convex cap and two semi-annulations which remain too shallow to prohibit the contained coenosarc from participating in the formative zone (Text-fig. 10F).

Both hydranth and gonangium rudiments follow a growth-rate curve to a final state of equilibrium or cessation of growth. On the reasonable assumption that the growth-curves are typical, then stage for stage the gonangium is larger than the hydranth, or at similar sizes is developmentally younger. This is borne out by a comparison of the final length and mass of hydranth and gonangium. It is also significant that at the same temperature the relatively large gonangium, as well as can be determined considering the vagueness of the developmental end-point, develops in approximately the same time as the hydranth.

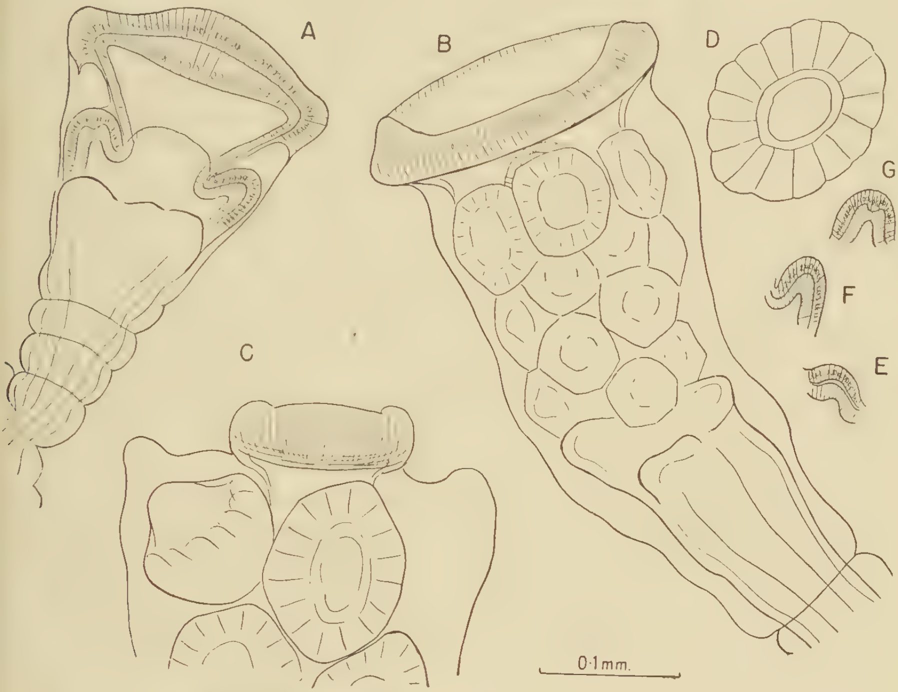
In essence, therefore, the gonangium in its early development may be regarded as a developing large hydranth, commencing to form a wide distal disk, and proximally a wide tapering enteron. If this interpretation is valid, why does not a gonangium exhibit more of hydranth structure, and why does not a hydranth produce medusae?

The answer to the second question is that during the critical development phase, the hydranth is not large enough for the initiation of medusa disks, and a second opportunity never arises since no growth occurs in the functional hydranth.

On the other hand, a developing gonangium forms medusa rudiments primarily because there is sufficient territory in its wall. This matter is discussed further in connexion with medusa formation and development. In the present connexion, what is important is the effect of medusa initiation upon the continuing development of the gonangium as a potential hydranth. Medusa initiation is precocious, that is, it occurs during the developmental period of the parent individual, and not later as in some other hydroids. The consequences are to be seen in the later development of the gonangium.

The epidermal terminal disk grows and forms a marginal ridge surrounding a central plateau (Text-fig. 10H), equivalent to the hydranth stage preceding tentacle demarkation. Without further change, however, it continues to grow, finally shrinking to a very considerable degree (Text-fig. 11A, B).

Up to the time of the initiation of the first group of medusae, the endodermis is in close contact with the terminal epidermis. If it remained in contact, further mutual growth might be expected to result in formation of tentacles and manubrium. As it happens, the terminal endodermis becomes drawn more and more into the formation and nourishment of the anterior medusae. The actual stage at which distal endodermal shrinkage becomes obvious is somewhat variable. In the example shown in Text-fig. 10G it is already very marked, and the gonangial cap is virtually epidermis alone. In the one shown in Text-fig. 11A, somewhat larger; the process has not yet commenced. Sooner or later, unable to serve two masters, the endodermis progressively abandons its contact with the epidermal plate, and the latter is clearly unable alone to develop tentacular or manubrial structure. Frequently the epidermis shrinks as rapidly as the endodermis, and the two layers remain in contact.



TEXT-FIG. 11. Development of Gonangium and medusae. A. Later stage with distal endodermis still in contact with epidermis, formation of distal ring of medusa buds and initiation of second ring proximal to it and progressive widening of stem coenosarc so that it becomes included in the developing gonangial unit. B. Late stage in gonangial development with distal epidermal disk at its maximum state, with successive graded rings of medusa buds. C. Distal part of gonangium at maximum size, showing pre-liberation medusae, and shrunken epidermal disk. D. Medusa at maximum size within gonangium, awaiting merely functional expansion. E. Initiation of medusa bud. F. Medusa bud rudiment. G. Medusa bud with epidermis thickening distally to form entocodon.

The gonangium is consequently to be regarded as a hydranth large enough to produce medusae but with its own later development aborted by the competitive demands of the medusae themselves.

MEDUSAE

The formation of medusae and the later development of the gonangium are too inextricably associated for separate treatment to be profitable.

The wall of the gonangium tapers in diameter from the wide distal disk to the narrow stem at the base. The first medusa buds to appear are four that develop in the same annular zone just below the disk where the wall is widest (Text-fig. 10G, H). By the time these are well established, the gonangium has undergone further growth, both in diameter at all levels and in length. In fact both the annulations of the stem and the contained coenosarc widen during the process, and the formative tissue of the gonangium clearly includes the coenosarc virtually as far as its junction with the main stem (Text-fig. 11A). As the zone immediately below the first four medusa buds attains a diameter or girth similar to the more anterior zone at the time of medusa-bud initiation, a second ring of buds is initiated (Text-fig. 11A). The process continues, without significant increase in distal diameter, but with a progressive lengthening of the whole unit and a progressive increase in diameter farther and farther down the cylinder and stem, with whorl after whorl of medusa buds becoming initiated as enlargement of the wall makes it possible (Text-fig. 11B).

The epidermal disk maintains its maximum size for a while (Text-fig. 11B), but finally it begins to shrink until once more it comes to lie around the endodermal component (Text-fig. 11 C). This is to be expected, for nutrition of the epidermis is maintained by the endodermis, and the earlier withdrawal of the endodermis left the epidermal cap in a state of chronic starvation.

Medusa buds of *Obelia* are comparatively small and are far from being the best material for the study of medusa morphogenesis. The essentials, however, can be determined and are in conformity with the earlier observations of Weismann (1883) and Agassiz (1862). The three earliest stages are shown in Text-fig. 11E, F, and G. A shield-like disk of tissue consisting equally of epidermis and endodermis, seen in profile in Text-fig. 11E, grows out from the parent wall and grows anteriorly as a deepening fold (Text-fig. 11F). It is notable that the rudiment develops from a relatively broad, flat area of wall, in contrast to the terminal growth of stolon, hydranth, and gonangium. There is no distinction into a distal cap and proximal cylinder. Also, the component cells of the whole are comparatively small and numerous for the size of the rudiment, indicating a relatively rapid rate of division. Finally, it is significant that the rudiments arise from wall tissue which itself is growing to a marked degree.

When the medusa bud has acquired a two-layered hemispherical form, the central distal region of the epidermis thickens. The rate of growth at the centre is or becomes greater than elsewhere and to this extent at least the development of the medusa bud resembles that of the gonangium, hydranth, and stolon. In this case, however, the epidermal thickening results in a proximal and not in a distal epidermal extension. The thickened region becomes two-layered and the inner layer is consequently segregated as the entocodon. This structure is the key to medusa development, as distinct from its initiation, and has been recently discussed at length elsewhere (Berrill, 1949). If no entocodon can be segregated, no medusa can be formed. Only the entocodal mass exhibits the tetraradial pattern uniquely characteristic of medusa organization. The details of its later development are not followed here, except to indicate the final size of the medusa attained before the functional expansion which occurs at the time of liberation. This is shown in Text-fig. 11D at the same scale as the rudiment. All medusae of this species are liberated with sixteen tentacles.

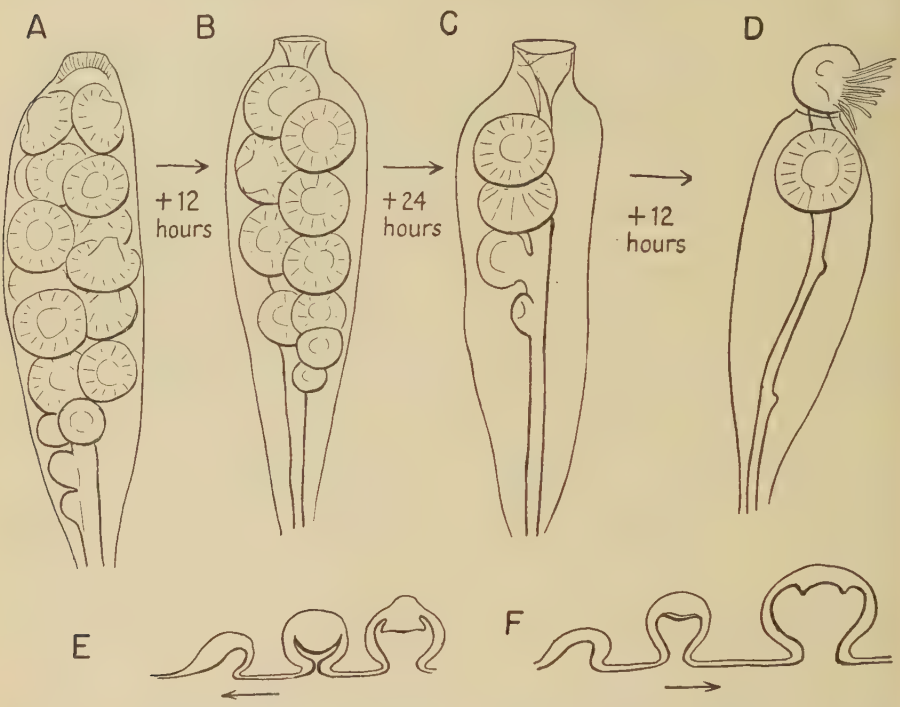
Within the limits of observational accuracy, it is notable that at a given temperature the time taken for a medusa to develop from initiation to liberation is approximately the same as the developmental periods of the hydranth, and of the gonangium up to its stage of maximal development as such (at 18–20° C. it is about 24 hours). It is also significant that in each of the three cases the final diameter at the end of its developmental period is approximately four times the diameter of the earliest definitive rudiment. If these two statements are accurate, and they appear to be reasonably so, we get the implications that the initial scale of organization determines the final size at the end of development, that the material in each follows a curve of growth representing a definite multiplicative value of its mass, and that each unit of mass grows to the same extent in the same time, and therefore that growth-rate and developmental time are independent of the initial total mass.

During the development of the medusae while attached to the blastostyle of the gonangium, regular contractions and expansions may be seen similar to the pulsation phases of stolon and hydranth. The time phases are similar, and there is the same alternating occlusion and dilatation of the hydrocoel (Text-fig. 12E, F). While this is probably due to a basic biological property of cells in a certain phase connected with growth, there is no doubt that in this case above all a useful function is performed, serving to draw nourishment from the hydroplasm streaming through the blastostyle, and effecting a regular metabolic exchange.

The hydroplasm streams in and out of the blastostyle hydrocoel in the same manner as it does in the developing hydranth. The contractions and expansions appear to be effected by the endodermis near the distal end. The result is that the developing medusae are maintained effectively and the stream is active usually until the last medusa has been liberated, although the innate pulsations of the developing medusae themselves probably are an adequate means of creating a communal feeding current.

When colonies bearing large numbers of large gonangia are kept under starvation conditions, so that any continued development must necessarily be at the expense of other tissues, the same phenomenon of differential resorption appears as that described by Huxley and de Beer for other hydroid species (1923) and for the ascidian *Perophora* (Huxley, 1921). Fully formed hydranths regress, developing hydranths complete development and then regress, while the medusa buds go on developing and finally all become liberated. Developing tissues are clearly in a very different physiological state from the same tissues fully developed. In this case the result is clearly both an inevitable one of colonial economics, and one of obvious value for the survival of the species. In fact, the medusa buds in completing their development may bring about the resorption of most of the stem coenosarc.

Liberation of medusae is effected not so much by the breaking of the medusa stalks down the length of the blastostyle, but to the continued growth in length of the blastostyle itself in a distal direction. As it continues to grow, the distal medusae are more or less pushed through the opening left at the gonangial tip by the shrinkage of the epidermal cap. As these break free, further extension carries the next group out, and so on until the last medusa is borne up to the threshold. The progression is shown in Text-fig. 12A–D.



Text-fig. 12. A–D. Liberation of medusae. A. Fully developed gonangium with cap at minimal state, with all medusae developed or initiated. B, C. Same gonangium 12 and 36 hours later respectively showing progressive elongation of stem or blastostyle distally, and successive liberation of medusae. D. Twelve hours later, with one medusa in process of detachment, and last medusa still within gonangium, with two abortive medusa rudiments on elongating residual blastostyle. E, F. Three stages of developing medusa buds showing contracted and expanded conditions; arrows show direction of hydroplasmic stream of blastostyle.

The liberated medusa immediately expands. There is no sign of gonads at this stage. It is equipped with 16 tentacles and 8 lithocysts. At 20° C. there are from 6 to 10 swimming contractions in about 3 seconds, followed by a rest pause of similar duration. The medusa rotates clockwise, completing a full circle with every 7 or 8 contractions.

SUMMARY

The growth cycle of *O. commissularis* McCrady is analysed in terms of tissue mass and shape, cell number and cell transformations.

A detailed analysis is made of stolonic growth typical of both free and attached stolons. Cells generally pass in succession through a phase of rapid non-vacuolate division, slower vacuolate division, a vacuolate recovery phase, a pulsation phase, and a resting phase. Cells in the resting phase may recommence the cycle at any time.

Chitin is almost certainly secreted by specialized glandular cells laden with highly refringent granules. Chitin is at first secreted as a viscous film, but rapid polymerization makes initial curvatures permanent features, such as annular rings. Local growth of epidermis in some way dissolves thick polymerized chitin.

All branches growing from a free or attached stolon and not forming at pre-existing junctions, undergo a phase of primary annular growth, a simple stolonic phase, and a second annular phase. Annulations are formed as the result of the epidermis rhythmically forming a series of terminal blobs in advance of the growing tip of the endodermis which penetrates them successively but belatedly. In the first series the blobs decrease in size until both tissue layers grow evenly as stolonic growth, the second series increase in size until a critical threshold size is attained which represents the hydranth rudiment.

The hydranth rudiment grows as a unit. Cells for the most part divide at the distal end to form a disk of increasing diameter. Cells are progressively added from the disk margin to the wall of the cylinder or body, where they pass through the pulsation phase and pull away from the surrounding perisarc. There is no further growth of the wall itself. The anterior disk becomes polygonal in outline, and the number of sides is always equal to half the number of tentacles to be formed, the tentacle number varying from 16 to 24.

Gonangia develop only from stem junctions already formed. Growth is annulated as in the case of the hydranth, but the epidermal blobs increase in size from the first and the final blob which represents the gonangium rudiment proper is somewhat larger than the comparable hydranth rudiment and is also relatively wider in the transverse plane.

The gonangium develops in the same manner as the hydranth up to the stage at which a hydranth would be about to form tentacle rudiments. At this time in gonangial development, the formation of medusa buds from the anterior body-wall immediately below the tentacle disk is so demanding that either one or both tissue layers of the anterior disk begin to shrink. The gonangium is interpreted as a relatively large hydranth whose later development is aborted by the precocious onset of medusa-bud formation.

Medusa buds arise from the gonangial wall while it itself is in progress of growth and arise in series as annular groups commencing anteriorly as the gonangial wall progressively attains an adequate girth.

The medusa bud arises directly from the wall and not from the terminal of a growing stolon as in hydranth and gonangium. The cells are small, indicating relative rapidity of division, and an entocodon, essential for medusoid organization, is formed by apical thickening of the medusa-bud epidermis, followed by segregation.

As medusae develop, the blastostyle to which they are attached grows and elongates towards the anterior end, thereby carrying medusae in effect through the distal aperture of the ripe gonangium, in order of their seniority. Upon liberation the medusae all have sixteen tentacles and no sign of gonads.

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On the Function of the Interstitium of the Testis

The Sexual Cycle of a Wild Bird, *Fulmaris glacialis* (L.)

BY

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With one Plate

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INTRODUCTION

BENOIT (1927, 1929) described two phases of the interstitial or Leydig cell of the testis of cockerels. He stated that in the embryo chick these cells become lipoidal in character. Later, in the young bird, they are 'rich in fat-bodies' and relatively poor in mitochondrial elements. Then, at the approach of spermatogenesis, a cellular transformation occurs until the Leydig cells have lost most of their lipoids and contain an abundant cytoplasm extremely rich in fuchsinophil substances. Benoit (1924) showed that the endocrine function of the testis remains unimpaired after the destruction of the tubule elements by Röntgen radiation, and presented quantitative evidence (Benoit, 1922) that the volume of interstitial cells runs hand in hand with the development of the secondary sexual characters.

Sluiter and van Oordt (1947) injected cockerels with gonadotrophic hormone and, with controls, demonstrated Benoit's two adult Leydig cell phases (lipoid and fuchsinophil). They did not observe Benoit's reported transition. They redescribe his two main types and report a third as well. These are:

1. The lipoid cell, which Sluiter and van Oordt believe not to be directly concerned with hormone production.
2. The non-lipoid cell, exhibiting numerous granular and filamentous mitochondria, which they term 'secretory cell A'.
3. Another non-lipoidal cell which contains large numbers of mitochondria and, in addition, one large vacuole which sometimes contains a crystalline substance. This is termed 'secretory cell B', and it evolves, they

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> say, from type 'A'. After injections of gonadotrophin the number of the non-lipoid cells rose sharply, and head appendages increased greatly in size. Sluiter and van Oordt believe that these 'secretory' cells produce the male sex hormone.

In the course of other studies of sexual periodicity the present author has been able to recognize the two chief cell-types of Benoit and Sluiter and van Oordt, but, working with wild species in which the sequence of sexual *behaviour* is known, he has had to disagree with Sluiter and van Oordt's interpretation of the function of the cell-types. The present contribution suggests that if the endocrine function is located in the interstitium, it is the lipoidal cells, and not the so-called 'secretory' non-lipoidal ones, which influence the bird in its prenuptial sexual behaviour. The species, *Fulmaris glacialis* (L.), used in this investigation does not develop measurable secondary sexual characters such as head-appendages. Its interstitial cytology, therefore, will be equated with its movements, its taking up of territory, and its display, copulation, ovulation, and moult. At present it is not denied that Sluiter and van Oordt's 'secretory' cells may possess some endocrine function, but it will be stressed that they appear in numbers only at and after the peak of spermatogenesis. Therefore they will be here termed *fuchsinophil* cells as distinct from the *lipoid* cells and *juvenile* cells (which will be described).

Material and Methods

Fulmaris is a petrel which ranges widely throughout the Arctic, North Atlantic, and North Pacific, and breeds plentifully in suitable areas in North Britain. It was chosen for the present study because it can be obtained with comparative ease during the periods of profound gonad modification, because it has a sharply defined single annual breeding season and fairly definite annual movements, and because its breeding habits and movements are well known and easy to check. It is not a true migrant, but a 'dispersive' species. In North Britain its behaviour may be broadly summarized as follows: in the last fortnight of August to early September the breeding population and young leave the nesting cliffs after the breeding season. They disperse over the sea. The first home-coming birds appear back on the breeding cliffs in November and December, dates of arrival varying from population to population. At first the numbers fluctuate, but by mid-February great numbers appear to be stationary in traditional breeding areas. Some breeding sites seem to be held by the same paired birds from the time of arrival (R. Richter, personal communication). From the time of their arrival the birds undergo a noisy display which probably has sexual significance. Apparent copulation has been observed as early as 6 April in a population where eggs were first laid about 17 May (L. S. V. Venables, personal communication). The incubation period is about 48–57 days (Richter, 1937; Fisher and Waterston, 1941). The young fly in August, and late in that month or early in September the whole population moves seaward. In the localities from which the majority of the present material was received (Scotland, Shetlands, Orkneys, and Bear Island) no birds usually remain about the cliffs throughout the whole year.

The material consists of testes from sixty-four fulmar petrels collected in the vicinity of nesting cliffs between January and late July. Birds taken before July were all from North British cliffs. The birds were shot and the testes dissected out and measured as soon as possible after death. As the birds were often collected in very awkward situations and dissected by voluntary helpers it was necessary to reduce fixation methods to basic simplicity. Thus, parts of each testis were fixed in formal-calcium and in Champy's fluid. Some formal-calcium material was embedded in gelatine and cut at 15 μ on the freezing microtome and submitted variously to sudan black with carmalum counter-staining; or to Scharlach R. with Ehrlich's haematoxylin counter-staining for the demonstration of interstitial lipoids and the revelation of adjacent tubule contents; or to the Schultz test for cholesterol. Other formal-calcium specimens were embedded in wax, sectioned at 7 μ and stained with Masson's triple stain for the determination of spermatogenetic stages, Leydig cell-counts, and the differentiation of fibroblasts from juvenile cells. The Champy material was wax-embedded, cut at 3 μ, and stained with Altmann's acid fuchsine and counter-stained with brilliant cresyl blue for the study of fuchsinophil elements. All measurements in μ refer to wax-embedded material unless otherwise stated.

In the present study only an estimate of the *relative* number of interstitial cells occurring during the winter period as against those occurring in the month when sperms first appear has been made. This has been done by counting the number of Leydig nuclei in fifty fields taken from the central zone of sections of typical testes. This method shows conclusively that a considerable increase in the number of Leydig cells occurs side by side with the maturation of the tubules.

THE CYTOLOGY OF THE INTERSTITIUM

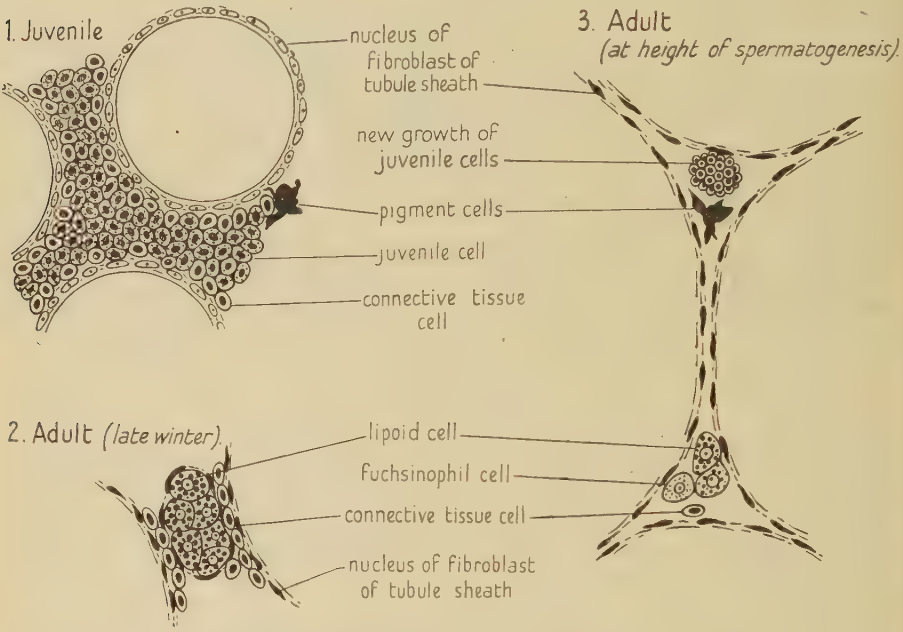
The interstitial cycle of this wild species with a clearly defined breeding season appears to be less complicated than that of the non-seasonal domestic cockerel. Sluiter and van Oordt give diagrams showing alterations in the proportions of lipoid cells and non-vacuolated and vacuolated non-lipoid cells in uninjected cockerels between the ages of 2 and 200 days. In wild fulmars such striking progressive numerical variation between lipoidal and non-lipoidal cells has not been observed except during a brief annual transition-stage.

Apart from the cells present in the intertubular blood-vessels, the following cells occur in the interstitium (see diagram, Text-fig. 1):

1. Fibroblasts of the sheaths of tubules, and in relation to blood-vessels and individual groups of Leydig cells.

2. Pigment cells (dentritic cells (Becker, 1927), or melanoblasts). These vary in number from bird to bird, and in the young, or in adults when the testes are sufficiently regressed, are sometimes compacted enough to give the whole organ a dark or at least dusky appearance.

3. Juvenile cells. These are generally rounded and from 7 to 10μ in diameter (nucleus 3μ). They appear to be the sole Leydig-type present at least throughout the first year. Their cytoplasm contains lipoid droplets. They become aggregated into groups but are not so markedly grouped as are the mature lipoid Leydig cells into which they later develop. Their mitochondria are much less evident than those of lipoid and fuchsinophil Leydig cells. In the interstitium of the young bird also occur cells of comparable size but which lack sudanophil substances.


1. Juvenile
nucleus of fibroblast of tubule sheath
new growth of juvenile cells
pigment cells
juvenile cell
connective tissue cell
3. Adult (at height of spermatogenesis)
2. Adult (late winter)
lipoid cell
fuchsinophil cell
connective tissue cell
nucleus of fibroblast of tubule sheath

TEXT-FIG. 1. Diagrammatic representation of the seasonal and age changes in the cell types in the interstitium of a wild bird.

4. Lipoid Leydig cells (in adult birds). These agree with the general description given by Benoit, and Sluiter and van Oordt, in the domestic cockerel. They reach a maximum size of about $20 \times 15\mu$ (nucleus 6–7μ), and are aggregated in groups in the sexually mature bird. By 'lipoids' are meant any of the chemically diverse fatty substances which colour with sudan black or Scharlach R.

5. Fuchsinophil Leydig cells (in adult birds). These agree with the 'glandular' phase of Benoit and the 'secretory cell A' of Sluiter and van Oordt. They are about the same size as lipoid cells.

6. Areolar connective-tissue cells. These, in the young bird, may be referable to the small non-sudanophil cells mentioned under 3. In the adult bird they are quite distinct from either lipoid, or fuchsinophil Leydig cell. They are non-lipoidal, and only 6μ in diameter (nucleus 4–5μ).

The Testis Cycle

1. *Immature Birds*

Odd birds taken in the vicinity of cliffs on which colonies were breeding had testes measuring from 15 × 10 mm. to 22 × 15 mm. In this species (as in many others) the left testis was almost always the larger in adults and yearling birds. During the breeding season the immature testis sometimes reached the maximum overall size, yet the tubules, containing inactive primary germ-cells, may measure only 35–40μ in diameter. Comparatively few elongated fibroblast cells occur per section. Small juvenile interstitial cells are conspicuous. It has not been proved that this latter type has a different origin from the non-sudanophil circular or oval cells which appear in older birds. The juvenile cells contain varying amounts of lipoid material which is dispersed unevenly through the cytoplasm in granules of varying sizes and which presents a mottled appearance. These cells contain very much less fatty substance than the large lipoid cells of adults at the height of winter (January). In the birds in question no mature Leydig cells are present. No mitoses are observed. As the species has a rigidly restricted annual breeding season the above individuals cannot be less than one year old.

2. *Mature Birds*

January (15th to 25th). The birds have come from the sea. A big population is assembled on and around the nesting cliffs. Testes of eight birds are nearly uniform, averaging 7 × 4 mm. in diameter. The maximum tubule-width is 70μ. Tubule-contents are limited to spermatogonia, two cells deep, which almost occlude each tubule. The intertubular tissue exhibits groups of large lipoid cells; each group is bounded by spindle-shaped connective-tissue cells. These lipoid cells mostly measure about 15 × 10μ with nuclei 4–5μ in diameter. The nuclei of the larger lipoid cells often have two prominent nucleoli. Surrounding the groups of lipoid cells are closely packed connective-tissue cells which are especially prevalent near the periphery of each testis. These smaller cells are not arranged in groups. Their diameter is limited to about 6μ and their nuclei are 4–5μ thick. Treatment by sudan black or Scharlach R. brings the lipoid cell-groups out in striking relief against the pale unstained tunicae propriae and tubule-contents. Pl. 1, fig. 1, shows this under low power. (Some of the tubules have been torn out by the freezing microtome, but the interstitium is not interfered with.) Pl. 1, fig. 2, shows a high-power view of individual groups in cross-section. Individual cells are so heavily laden with lipoids that a great deal of differentiation is necessary before the nucleus can be seen clearly in a 15μ section. After Champy-fixation and wax-embedding the lipoids are nearly all lost, but Altmann-staining reveals abundant fuchsinophil particles resembling mitochondria among the globular network which remains after the lipoids have dissolved. Schultz's test for cholesterol is positive in the lipoid cells, proceeding so strongly as to obliterate all cellular detail. It is negative for the tubules.

Blood-vessels are very obvious in the neighbourhood of the lipoid groups. No mitoses were observed. There are about 17 nuclei of lipoid cells per microscopical field of view (average of 50 fields) in testes measuring 6 × 5 × 5 mm.

February (first week). Seven testes average 9 × 6 mm. Tubules now measure up to 110μ in diameter. They still contain mostly spermatogonia with a few primary spermatocytes in synizesis. Two birds contain many spermatocytes in synizesis and up to six zones of germinal cells in a tubule. Connective-tissue cells are abundant with nuclei measuring 5 × 4μ. Partial dispersion of the lipoid cells, beginning at the onset of tubule expansion, can already be seen. One tract of lipoid cells was squeezed out to a length of 170μ (measurement after formal-calcium fixation) in varying width. The lipoid cells are highly sudanophil—it is impossible to see more than the outline of the nuclei after colouring a 15μ section with Scharlach R. After Champy-Altmann treatment the lipoid cells exhibit mitochondria in the network between the empty globules. Some compressed lipoid cells measure 25 × 8μ and contain nuclei 7 × 5μ. Nuclei of the spindle-shaped fibroblast are now 15μ long. No fuchsinophil cells are observed. Blood-vessels are very prominent. No mitoses are seen.

February (last week). Five testes average 12 × 9 mm. A sharp and substantial increase in volume has occurred in the space of about a fortnight. Tubules have increased up to 150μ in diameter. All testes show maturation division and three contain spermatozoa arranged in bunches, with a few mature sperms free in the lumina. The intertubular tissue presents equally remarkable changes. Extensive empty tracts appear. The initial impression given is that hand in hand with the upsurge of spermatogenetic activity there has been a large-scale reduction in the number of Leydig cells. The intertubular tissue in a given section now generally consists of odd strands of connective tissue, prominent blood-vessels, and a few isolated groups of Leydig cells. The reason for this appearance is that the expanded tubules have dispersed the once closely packed lipoid groups. Blood-vessels have become more obvious because they are no longer partially concealed by packed masses of Leydig cells and because they retain their continuity and stretch through the apparently denuded interstitium. Isolated groups of lipoid cells still colour deep scarlet with Scharlach R. (Pl. 1, fig. 3). Although spermatogenesis has proceeded a long way there is no visible evidence of any transfer of lipoids to the tubules for the nourishment of the developing sperms as claimed by Stieve (1923, 1926) in the goose. Lipoidal cells are nearly all at or near maximum size. They show an abundance of fuchsinophil elements after lipoid dissolution and Champy-Altmann techniques. Many connective-tissue cells are still present. Although spermatozoa have appeared, some testes are so heavily lipoidal that slides appear mottled red (after Scharlach R.) if held to the window and examined with the naked eye.

March (first week). Seven testes average 14 × 10 mm. Tubule contents are in much the same condition as in birds taken a week before. Although the dispersed lipoid cells turn deep scarlet or black with fat-colouring reagents, no trace of lipoid is detected in the neighbouring tubules near the prominently bunched spermatozoa, though von Ebner's granules are faintly sudanophil, and give a faintly positive Schultz reaction. Lipoid cells now measure up to $20 \times 15\,\mu$ with nuclei $7\,\mu$ thick and nucleoli $1{\cdot}5\,\mu$ across. Some lipoid cells still measure only $10 \times 6\ \mu$, however. Areolar connective-tissue cells are also present. Counts in the largest testis ($15 \times 10 \times 10$ mm.) reveal an average of 7 nuclei per field (50 fields counted). There has been a gain of more than 300 per cent. in Leydig cells since January.

April (third week). Three testes, of which two measure 15×10 mm. and the third 24×12 mm. The two first-mentioned testes have tubules about $160\,\mu$ in diameter. These contain vast numbers of mature, bunched sperms, with some free in the lumina. Lipoid cells are maximal in size and are as strongly sudanophil as in the previous months. There is no evidence of a movement of lipoid into the tubules. The only tubule lipoids are the faintly sudanophil von Ebner's granules. Mitochondria show in Champy-Altmann-treated lipoid cells. Maturation divisions are occurring abundantly in the tubules, but mitosis is not observed in the interstitium.

The largest testis of the group (24×12 mm.) presents a different picture. Its tubules measure up to $200\,\mu$ wide. Sperms are free in enormous numbers in some lumina but are still arranged in bunches in the less-advanced peripheral tubules. Only in these outer tubules is spermatogenesis still proceeding actively in the primary and secondary spermatocyte stages. The presence of numbers of degenerating cells in some lumina shows that those tubules have passed the peak of spermatogenesis. Although there is no sign of lipoids in the tubules, the lipoid content of the interstitium cells is being depleted. Lipoid cell nuclei are generally seen more readily than before after Scharlach R. colouring, and some lipoid cells appear to lack sudanophil substances altogether. After Champy-Altmann techniques fuchsinophil elements show as in previous months, but two new appearances are now commonly present. These are:

1. Cells having the same general size and appearance as the largest lipoid cells but lacking the characteristic globular network which appears after the lipoids are lost during embedding in wax. The nucleus is of maximal size and the nuclear cytoplasm has dark granules scattered unevenly through it. The general cell cytoplasm is speckled profusely with fuchsinophil elements which completely hide any suggestion of a globular network which may have remained. There are globular networks in adjacent cells. In one group of three lipoid cells a differential mitochondrial appearance occurs. The more heavily fuchsinophil cells appear to be referable to the 'secretory cell A' of Sluiter and van Oordt. The above observations go towards substantiating Benoit's claim that lipoid cells develop into cells which are heavily fuchsinophil. It must be stressed, however, that the present observations refer only to a few cells in the testis at the height of spermatogenesis.

2. There appears densely packed between two peripheral tubules a crop of

small cells very like the juvenile type. They possess nuclei about 4μ in diameter, and their scanty cytoplasm appears only faintly granular after sudan-colouring. There is at present no evidence as to the precise origin of these cells.

May (third week). The first egg of any colony under observation was seen on 13 May and a fully formed egg was dissected out of a female on the 15th. The testes of six birds of this period average 12 × 8·5 mm., ranging between 15 and 10 mm. in length. Tunics are now very variable in thickness. This is caused partly by the presence of voluminous blood-vessels, including veins 90μ thick—a tremendous increase in bore over those of pre-spermatogenetic months. All testes examined contained sperms but it is obvious that in the case of male fulmars the height of the season has passed. The tubules generally contain aggregations of degenerate cells and mitotic activity is mostly restricted to the extreme peripheral zones where a few spermatocytes are still dividing. The interstitium of the larger testes still contains lipoid cells but fewer with the typical network after Champy-Altmann treatment. Quite often large cells are filled with a granular fuchsinophil cytoplasm instead of the former lipoids. There are several interstitial tracts filled with smaller cells of the next Leydig generation. Also noteworthy is the presence of numerous almost empty tracts measuring up to 40 × 150μ which follow the contours of the shrinking tubules. Blood-vessels, connective-tissue fibres, and rounded or oval fibroblasts pass across them.

June. Three testes average 10 × 6 mm. Tubules are now 110μ in diameter. This is a period of profound testis regression and reorganization. In the tubules a remarkable new epithelium has appeared. This is at first composed of a single layer of large cells with nuclei 5μ in diameter. In some tubules two layers have appeared and altogether the epithelium may be 30μ thick. Isolated in the centre of many of the testis lumina are masses of degenerate germ cells up to 35μ thick, and some lumina are completely choked with these degenerating products of the former spermatogenesis. In some testes, however, some tubules are already almost free of debris.

The tubules of the testis have meanwhile become lipoidal and react positively to Schultz's test. The degenerating cells interior to the new epithelial generation are heavily sudanophil and the sloughed degenerating material somewhat less so. The new basal layer of epithelial cells is sudanophil to a varying degree. In some testes the cells are heavily lipoidal near their free borders; in others only a meagrely sudanophil granular mottling is retained.

The interstitium too is in a regenerative phase. The former generation of Leydig cells has disappeared and the interstitium is filled with a new generation which resembles the juvenile cell and which first made its appearance in the largest testes of late April at a period when fuchsinophil cells first became plentiful and the most-advanced tubules exhibited signs of epithelial degeneration. The smallest cells between the tubules in June are only faintly lipoidal. At odd places in the testis they still remain aggregated together in tracts

100–200μ long. In these tracts of undispersed new Leydig cells there also occur cells which are larger and more heavily lipoidal, and which measure $10 \times 6\mu$ with nuclei 4μ in diameter. These are not organized into groups. Elsewhere in the interstitium the lipoid Leydig cells have reached maximal size and are grouped. The groups are bounded by spindle-shaped fibroblasts. After Champy fixation and Altmann staining, numerous minute mitochondria are observed. These very closely resemble Sluiter and van Oordt's fuchsinophil type. The interstitium also contains pigment cells. Arterioles 30μ in diameter, with a bore of about 15μ, and veins of the same volume, also transect the interstitium.

Late July (15 specimens). Testes average 7×5 mm. These birds were collected on Jan Mayen and Bear Islands in the Arctic during 1947 and 1948. They were taken in the vicinity of the nesting cliffs after the young were hatched. Each bird used in the present study showed a 'brood patch' which probably indicates that it has reproduced (in *Fulmaris* the sexes take turns at incubating the eggs). Each bird was now moulting. These testes present a uniform picture except that the new tubule-epithelium, consisting of two layers of spermatogonia, is slightly uneven in its sudanophil properties. No tubule, however, retains sufficient lipoids to exhibit a large vacuole after wax-embedding. Tubules are regressed to 50μ and are occluded by the new germinal generation; degenerated remnants of the last spermatogenesis have been entirely eliminated. The interstitium is more or less heavily lipoidal in all birds, with the Leydig cells generally organized into groups which are dispersed evenly. Several testes, however, show tracts of meagrely sudanophil small Leydig cells apparently in the process of becoming lipoidal. These are still retained undispersed in restricted areas. The mature Leydig cells, and many of the smaller ones, show mitochondria. Pigment cells measure about $20 \times 5\mu$ and their nuclei may be seen without bleaching in Champy-fixed and Altmann-stained material.

Both the tubules and the interstitium are now almost completely regenerated (Pl. 1, fig. 4).

Late July (2 specimens). Non-breeding birds with no brood patch. These reveal a testis-type which does not fit into any group so far described. Testes measured 6×4 mm. and those of both birds were dark with concentrated pigmentation. The tubules were 70μ wide and contained primary spermatocytes.

Had it not been for their curious interstitium these birds would have passed for winter adults. The interstitial cells were mostly of the juvenile type measuring $7 \times 5\mu$ with nuclei $5 \times 4\mu$ thick. While the nuclei were still small some of these cells had become more strongly sudanophil, and many, in the centres of the wide interstitial tracts, were now approaching adult size ($24 \times 10\mu$ with nuclei 6μ wide). Each testis gave the impression that more and more juvenile cells were becoming heavily lipoidal until big groups of almost mature Leydig cells, bounded by spindle-shaped connective-tissue cells, had appeared (Pl. 1, fig. 5).

The above testis is nothing like that of the July adult, and quite unlike that of the yearling bird. A comparable appearance has been observed (Marshall and Coombs, unpublished) in the rook at a period when the 16-months-old young was changing by moult to its first adult plumage. (The age is known from details of plumage.) As the testis of the fulmar yearling presents an entirely different picture, it may be that the present birds are just over two years old and will breed the following year.

DISCUSSION

In sexually immature fulmars a small partially lipoidal *juvenile* cell occurs in the intertubular tissue of the testis. This cell develops into the large and strongly sudanophil mature *lipoid* Leydig cell not before one year and possibly when the bird is just over two years old. Once sexual maturity is reached, the bulk of the Leydig cells appear to belong to the lipoid type throughout the year except for a brief period which begins about the height of spermatogenesis. From the time the sperms have begun to appear, the Leydig cells are seen to be losing their lipoids until only a faint mottling of sudanophil substances remains in the cytoplasm. At this period numbers of non-lipoidal or partially lipoidal and strongly fuchsinophil Leydig cells appear. At the stage when free sperms are visible in the tubules and degenerating epithelial products begin to appear, a Leydig cell regeneration begins at odd spots in the interstitium. At the time of testis collapse and an accompanying fatty metamorphosis of the tubular epithelium, there appears a massive new development of small Leydig cells which may be identical with the juvenile cell in the young bird. These soon become partially lipoidal. They become distributed through the denuded interstitium, gain in size and sudanophil content, become organized into groups bounded by fibrous connective tissue, and so the interstitial cycle has begun once more. The testis is still at its minimal post-nuptial size and apparently in the 'inactive' phase (of various authors) unless the interstitium is studied with appropriate fat-colouring reagents. Meanwhile, under the fatty metamorphosis, the tubule-epithelium has become regenerated and a curiously uniform new generation of epithelial cells has been laid down. These are now meagrely sudanophil only near their free borders. The tubules, too, are now practically free of debris from the former spermatogenesis and the testis is apparently completely rehabilitated. The young of the current breeding season are still in the nests. An anatomical basis for an internal rhythm may thus have been shown. It will be recalled that Bissonnette and Wadlund (1931) report that their light-stimulated starlings could only maintain spermatogenesis for a certain period after which regression began.

While the above cycle is proceeding (when the Leydig cells are predominently lipoidal), the fulmar population has come in from the sea and is established in vast numbers on its breeding cliffs in December. This movement to the breeding territory occurs while daylight is decreasing. Between January (when its Leydig cells appear to be uniformly lipoidal and when the testes average only 7×4 mm.) and mid-May (when the first eggs are laid) the birds take up territory, perform sexual display, select egg-sites, and follow the usual pattern of pre-nuptial behaviour which is generally considered to be under neuro-humoral control (Marshall, 1936). Meanwhile spermatogenesis is completed and copulation occurs. It is necessary to attempt to relate these factors, and to try to bring the experimental work of Sluiter and van Oordt and the results of other workers into line with the cycle outlined above.

In *Fulmaris* the testis measures about 15×10 mm. in early March, when free sperms have appeared in the lumina. Although the testis is still far below its maximal size its volume has increased greatly. With this overall enlargement there is a decrease in the thickness of the testis tunic and a considerable increase in the diameter of the tubules, which accommodate the developing germinal elements. As the tubules expand the tightly packed Leydig cells disperse, creating an appearance, in any given section, of a general decrease in interstitial cells at the time of spermatogenesis. This is a false appearance: lipoid Leydig cells increase by at least 300 per cent. between mid-winter and the time of spermatogenesis. The present study therefore agrees with those of Benoit (1929), Groome (1933), and Sluiter and van Oordt (1947), who state that the Leydig cells of birds increase in number with heightened sexuality.

The *Fulmaris* material makes it clear that in the interstitium of young birds there occurs a meagrely lipoidal cell which develops into a mature lipoidal Leydig cell, a process also noticed by Benoit. It is the next stage in the cycle which remains still in doubt, and the matter is inextricably bound up with the question of the relative function of the lipoid and fuchsinophil Leydig-cell types. After sperms have appeared free in the tubule lumina, most Leydig cells are still too densely sudanophil to show even the nucleus clearly in a 15 μ section. From now onwards, however, a steady diminution of the lipoid content takes place. At the time when the testes are about to collapse, most of the lipoid cells show only granular sudanophil substances in their cytoplasm, and even taking tubule-expansion into consideration it is evident that their numbers have decreased. Stieve (1926) noted the decrease in lipoid content in Leydig cells and published a coloured plate (p. 15 of the above reference) illustrating his theory that Leydig cells are trophic in function and give up lipoids to nourish the developing germinal epithelium. Occasionally during the present investigation frozen sections coloured with Scharlach R. appeared to agree with Stieve's illustration in that they showed a scarlet mottling appearing dispersed among the spermatogenetic products. On repetition, however, it was found that the above appearance, in *Fulmaris*, had been caused by errors in technique; interstitial cells had been ruptured and small coloured lipoid droplets had become lodged in the neighbouring tubules of the section. No sudanophil substance was detected in the tubules until mature sperms appeared. At this stage von Ebner's granules exhibit a lipophanerosis and are faintly but unmistakably lipoidal.

Benoit declared that the lipoid cell loses its sudanophil contents and develops into a fuchsinophil cell. Sluiter and van Oordt failed to confirm this transition, and left the question open. In *Fulmaris* the lipoid cell (after Champy-Altmann techniques) often shows abundant mitochondria among the network between empty vacuoles from which the lipoids have dissolved. At about the height of spermatogenesis almost identical cells occur immediately adjacent to the lipoid cells; these differ only by the possession of a more profuse development of mitochondria. It is believed then that Benoit's view was correct. Benoit did not observe Sluiter and van Oordt's vacuolated fuchsinophil cell ('secretory cell B'), into which the latter authors believe the non-vacuolated fuchsinophil cell develops. This 'cell B' of Sluiter and van Oordt has not been observed in *Fulmaris* material, but something like it has been seen by the present author in other species.

Sluiter and van Oordt declare that the vacuolated fuchsinophil cells 'undergo a regression and pass over into lipoid cells'. Of this, no confirmation has been found in *Fulmaris*. Between the two generations of Leydig cells intervenes a new appearance of small meagrely lipoidal cells which spring up at odd points of the interstitium after the most-advanced tubules have shed sperms and while the less-advanced tubules still contain dividing spermatocytes. This new Leydig cell generation appears abundantly during the period of tubule breakdown. A big tract of small cells shows only a faint tinge of sudan colouring when they are very young and so for a time the interstitium is almost totally devoid of lipoids. At this stage, however, fatty metamorphosis is proceeding in the tubules. The tubules become brilliantly sudanophil. Rowan (1929) has evidence of this tubule-metamorphosis, describing tubal 'vacuities'. Rowan mentioned that the 'vacuities' look as though they had been artificially produced, but emphasizes that they are not artifacts. He was right; they are the empty spaces left after the fats were dissolved out during alcohol grading. Bissonnette (1930) also mentioned the 'vacuolation' of the epithelium in the regressive testis, apparently without realizing its significance.

It is suspected that the new Leydig generation arose from the old generation of exhausted Leydig cells but no proof of this can be offered.

The present work does not substantiate the opinion of Rowan and Batrawi (1939) that the winter interstitium consists solely of spindle-shaped connective-tissue cells with heavily staining nuclei, nor can any confirmation be offered of Bissonnette and Chapnick's (1930) suggestion that the interstitial cells may disappear in the spring by being incorporated in the tunicae propriae as they distend to accommodate the ripening tubule-products.

We now come to the question of the relative function of the two cells: lipoid and fuchsinophil. Sluiter and van Oordt injected cockerels with gonadotrophin obtained from pregnant mares' serum and attempted to equate the resultant accelerated growth of the birds' head-appendages with the cytology of the interstitium. They report that the total number of all three interstitial cell-types (lipoid, fuchsinophil, and vacuolated fuchsinophil) increased enormously as the head-appendages developed. In normal, uninjected birds about 50 per cent. of this increase in number is due to the increase in lipoid cells. In birds treated with gonadotrophin only the fuchsinophil cells increased in number. Sluiter and van Oordt therefore term these 'secretory cells'. Because the head-appendages of gonadotrophin-treated birds increase in size along with the increase of non-lipoid cells, the above authors declare that it is obvious that the lipoid cells are not directly necessary for the production of hormone. They showed that the lipoid cells contain cholesterol derivatives, but believe that although the lipoid cells cannot be directly concerned with the production of androgens they may act as storage cells of 'special elementary substances'.

In *Fulmaris* wherever the Leydig cell is positive to sudan black or Scharlach R. it gives a positive Schultz cholesterol reaction. Later in the season, when the lipoid cells have lost much of their sudanophil content, only a faint blue-green mottling appears when the Schultz test is applied. It has not been possible to locate a non-lipoidal fuchsinophil cell in any section (i.e. formal-calcium-fixed) to which Schultz test can be applied.

In other parts of the testis the rule holds: if a section of tissue colours with Scharlach R. then it will be Schultz positive. This extends to the metamorphosing tubules, von Ebner's granules, and the regenerating epithelium of the vasa efferentia after the breeding season.

Although the biochemistry of the sex hormones *in vivo* is still obscure, their derivation from cholesterol in nature appears to be a comparatively simple process. Like the present investigator, Sluiter and van Oordt established the presence of the probable precursor of the sex hormone in their 'storage' cells but were not able to do so with certainty in their 'secretory' cells.

Apart from its richness in cholesterol, the present author believes that the lipoid phase of the Leydig cell is the primary producer of sex hormone. Lipoid cells increase in number during heightening sexuality in the wild bird, and are the only apparently active cell-type present during the period when the birds flock back to their nesting cliffs, take up territory, display to each other, take up nest-sites, and achieve spermatogenesis. With the appearance of abundant spermatozoa, completely fuchsinophil cells appear in quantities, and it is not denied that they may possess some glandular significance. The present author, however, inclines to the idea that they are merely a phase of the endocrine lipoid cell and that Sluiter and van Oordt's injections perhaps stimulated and accelerated the naturally slow evolution from juvenile→lipoid→fuchsinophil cell. It could be that the appendage growth in the cockerels came from the stimulated lipoid cells, which, having discharged their endocrine function, passed over to the fuchsinophil phase.

Sluiter and van Oordt, while not agreeing with Stieve (1923, 1926) that the lipoid cells are solely trophic in function, consider them primarily storage-cells and state that their number depends on the nutritive conditions of the animal. In *Fulmaris* there is no evidence that this is so. Again, Sluiter and van Oordt suggest that lipoids sometimes occur in such quantities as to leave no room for a glandular function in the cell. Wells (1925), however, has evidence that the liver cells of man (especially among alcoholics) may sometimes contain so much fat it is difficult to find any cell cytoplasm, yet there is no clinical evidence of any impairment of function.

Sluiter and van Oordt state that many vacuolated fuchsinophil cells in cockerels older than 2 months 'undergo a regression and pass over into lipoid cells' and that in the adult cock 'only a small percentage of the original secretory cells still function as such . . . the others have become storage cells'. In *Fulmaris* a new meagrely lipoidal generation, described above, comes after the advent of fuchsinophil cells and before the next appearance of large lipoid Leydig cells. The only way in which we can bring the above opinion of Sluiter and van Oordt into line with the present work is to think of their mitochondrial 'secretory' cells as the new generation of partially lipoidal Leydig cells which do, in fact, also exhibit mitochondria. In any case, the gaining of lipoids, far from being a 'regression', is actually another active phase in the cycle. The interstitium, in short, is once more becoming lipoidal in preparation for the forthcoming activity connected with the next breeding cycle.

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Summary

1. An investigation of the seasonal cycle of the interstitium of the testes of birds, based on sixty-four individuals of varying age, has been carried out on the fulmar petrel, *Fulmaris glacialis* (L.).

2. The following cells (see diagram, p. 268) occur:

(i) Fibroblasts of the sheaths of tubules, blood-vessels, and of groups of Leydig cells.
(ii) Pigment cells.
(iii) Juvenile Leydig cells (generally containing lipoid droplets).
(iv) Lipoid Leydig cells.
(v) Fuchsinophil Leydig cells.
(vi) Areolar connective-tissue cells.

3. In the young bird the juvenile cell develops into the lipoid Leydig cell at a time when the testis-tubules also indicate approaching sexual maturity.

In the adult, the interstitium generally consists for the most part of lipoid Leydig cells. These exhibit mitochondria when the soluble lipoids are removed by embedding in wax. At the height of spermatogenesis, and at the beginning of epithelial breakdown and tubule-regression, most of the Leydig cells have lost much of their lipoids and some of them exhibit increasing amounts of fuchsinophil substances, as described by Benoit. A fuchsinophil cell thus appears.

4. The Lipoid cell at all seasons shows a positive Schultz reaction (for cholesterol), which corresponds in intensity to the amount of sudanophil material present. It has not been possible to demonstrate cholesterol in the fuchsinophil cell.

5. At the time when the tubules are at their maximum diameter and their degeneration is under way, mitochondria are at the greatest abundance in the Leydig cell. At this period a new generation of Leydig cells arises in the interstitium. These cells quickly become meagrely sudanophil and resemble the sudanophil juvenile cells of the immature bird. They are Schultz positive. The tubules collapse; the new Leydig generation fills the empty interstitium. They exhibit profuse mitochondria, gain in lipoid content and so the interstitium is regenerated.

6. The exhausted tubules undergo a fatty metamorphosis at the time of their collapse; at the period when the interstitium has little lipoid the tubules are full of it. Beneath this fat (also Schultz positive) arises a new tubule-epithelium. The tubules as well as the interstitium are regenerated while the young of the next generation are still in the nests. An anatomical basis for an internal physiological rhythm may thus have been shown.

7. The new interstitial cells are already heavily lipoidal and the new tubule epithelium contains spermatogonia when the petrels move seaward away from the breeding cliffs in the autumn. They return to the breeding area in November and December. Thus testis regeneration, and movement both away from and back to the breeding area, occurs while the days are getting shorter.

8. From the time the birds appear on the breeding cliffs (and apparently since the autumn) there is an increase in lipoid cells along with heightened sexuality as revealed by the ripening tubule-products. These supplementary lipoid Leydig cells seem to develop from the small non-sudanophil areolar connective-tissue cells which are prominent in the adult interstitium.

9. Whilst it is not denied that the fuchsinophil cell ('secretory cell' of Sluiter and van Oordt) may have an endocrine function, the present results suggest that the lipoid Leydig cell is the primary secretory component of the avian testis. When the interstitium, after reproduction, passes once more to a lipoidal phase, it is not losing its secretory function as Sluiter and van Oordt infer, but is regenerating in readiness for the next season's breeding activities.

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EXPLANATION OF PLATE I

Fig. 1. Adult winter testis (January). Low power, showing a. Lipoidal interstitium; b. Tubules free of lipoids; c. Tunica albuginea. (Sudan black and carmalum at 15 μ.)

Fig. 2. Ditto. High power, showing a. Individual group of lipoid Leydig cells; b. Tubule containing spermatogonia. Top tubule has been removed by freezing microtome. (Sudan black and carmalum at 15 μ.)

Fig. 3. Adult pre-nuptial testis (late February) showing a. Group of lipoid Leydig cells; b. Pigment cell; and c. Spermatozoa. (Scharlach R. and Ehrlich's haematoxylin at 15 μ.)

Fig. 4. Adult post-nuptial testis (late July) showing a. Regenerating lipoidal interstitium; and b. Last remaining lipoids of fatty metamorphosis in tubules. (Scharlach R. and Ehrlich's haematoxylin at 15 μ.)

Fig. 5. Change-over phase (late July) from juvenile to mature lipoid Leydig cells. a. Indicates group of cells which have become large and lipoidal; b. Shows undifferentiated juvenile cells. (Sudan black at 10 μ.)

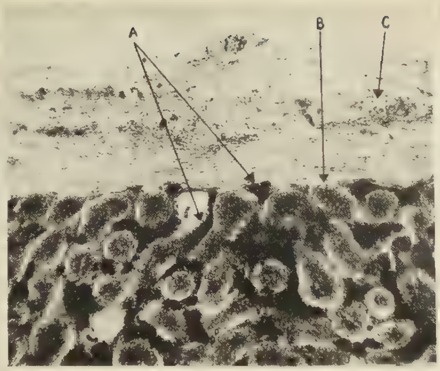

FIG. 1

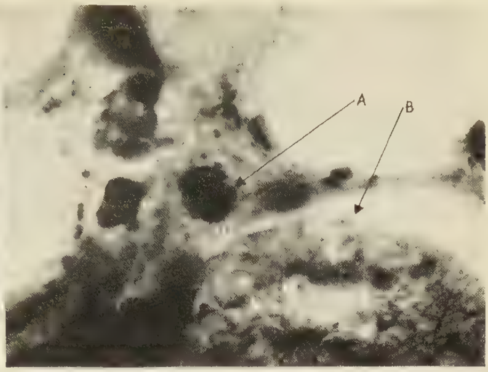

FIG. 2

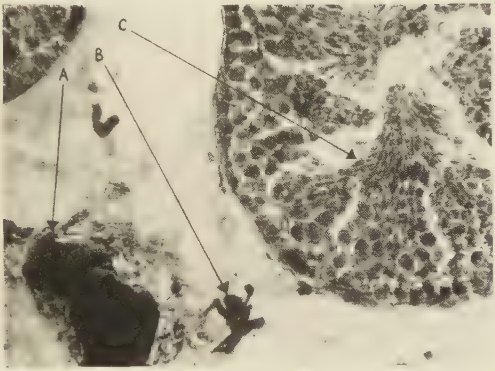

FIG. 3

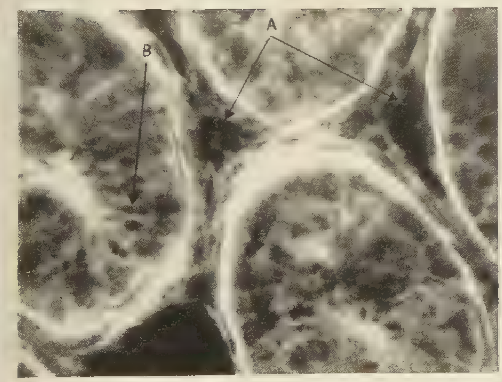

FIG. 4

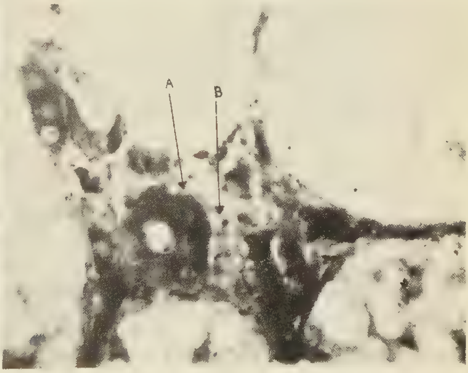

FIG. 5

A. J. MARSHALL—PLATE I

The Mechanism of Insemination and the Mode of Action of the Spermatophore in *Gryllus domesticus*

BY

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INTRODUCTION

THE literature on the different types of spermatophores in Orthoptera is voluminous, yet detailed accounts of their function are lacking. Spann (1934) showed that the transference of sperm enclosed in spermatophores was observed by the early writers such as Siebold and by Lespes (1855). Since then descriptive accounts have been published by a number of authors, the most important of which are Boldyrev (1912–14) and Gerhardt (1913–21).

Regen (1924) working with *Liogryllus campestris* brought to light the mechanism by which a spermatophore is evacuated. His description cannot, however, be applied to *G. domesticus*. This might be attributed either to structural variation or to his having overlooked certain details which would necessitate some modifications based on the study of the house cricket spermatophore.

G. domesticus L. has proved to be ideal material for this study. A couple can be brought to copulate in a limited space and a young male can produce, on a hot day, two or three spermatophores.

THE STRUCTURE OF THE SPERMATOPHORE

The spermatophore of *G. domesticus* (Text-fig. 1) is composed externally of three parts: the ampulla, the handle, and the spermatophore tube. When the spermatophore is in the mould, which is at the hind end of the male's body, the ampulla is found lying on the last sternum covered on each side by a lateral muscular flap (Text-fig. 2). The handle extends forward, fitting into the floor of the spermatophore mould. The tube, passing through the handle, bends on itself and extends posteriorly, lying in a very thin sclerotized grooved rod (Text-fig. 2). The convex surface of the handle will, therefore, mark the ventral surface of the spermatophore.

The ampulla (Text-fig. 1). This is a globular structure with a teat-like projection at its posterior end, the papilla. It varies in colour and consistency according to how long it has been ejected. When it is first ejected it has a white milky appearance and a very soft viscous consistency. About one hour later it becomes glassy, and a brown colour appears at the posterior end and extends a little way down the sides along the inner surface of the inner layer (Text-fig. 3*d*). It then becomes very hard and brittle.

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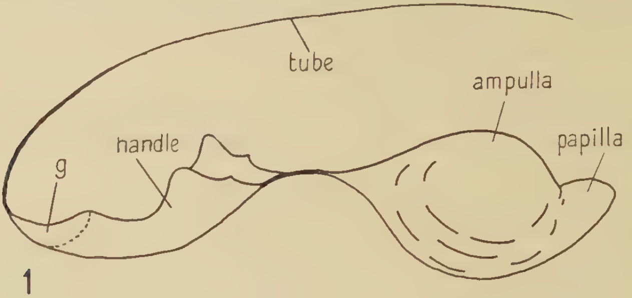
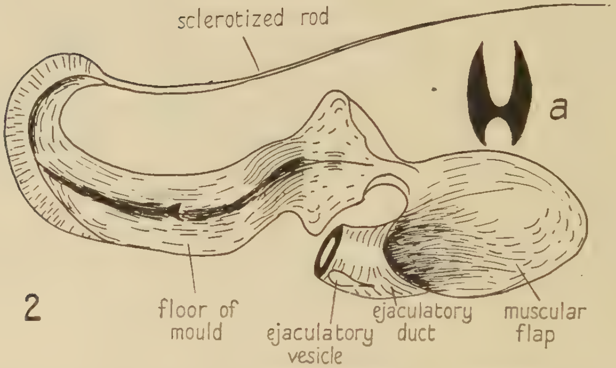
The wall consists of four clearly distinguishable layers (Text-fig. 3).

1. The outer layer is a thin transparent membrane becoming comparatively thick at the posterior end of the ampulla and round the papilla. In a recently ejected spermatophore it is a smooth membrane and gives the ampulla a spherical shape. In an empty spermatophore, however, or in one that has been kept in the mould for a fairly long time in dry air, the membrane is no longer smooth but shows many folds as it collapses on the inner layer. In sections it appears to be composed of several lamellae.





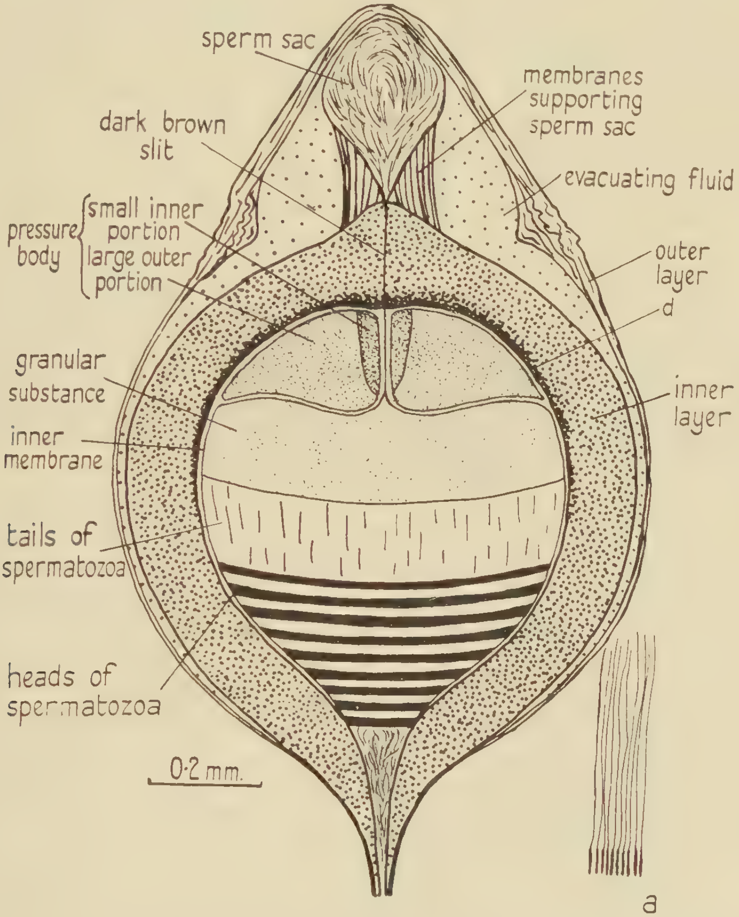
TEXT-FIG. 1. The spermatophore of *Gryllus domesticus*. *g*, gelatinous white material covering the invagination at the end of the spermatophore handle (marked with a dotted line).

TEXT-FIG. 2. The mould of the spermatophore. The second muscular flap has been removed. *a*, a transverse section through the sclerotized rod.

2. The evacuating fluid fills the gap between the outer and the inner layer. This gap widens posteriorly where the fluid increases in quantity, and narrows anteriorly as the outer and the inner layers approximate. When a smear of this fluid was left to dry on a slide a very thin transparent film remained. When a drop of Millon's reagent mixed with glycerine was added and the slide heated a very faint red colour appeared. The ninhydrin reaction was also positive; so it is most probable that this fluid contains protein. At a rough estimate 0·001 c.c. of this fluid is present round the ampulla. In an empty

spermatophore or in one which has been left to dry, the fluid disappears, the outer layer collapses and the ampulla loses its even surface.

3. The inner layer is the most conspicuous layer owing to its hardness and thickness (about 0·17 mm.). It is very firm and brittle, and maintains its shape in a filled or empty spermatophore. The hardening of the ampulla is entirely attributable to certain chemical changes taking place in this layer as the ampulla is exposed to the air. A newly ejected spermatophore could be squeezed between a slide and a coverslip, and the granular nature of this layer could be easily seen. Later, however, it becomes a hard homogeneous transparent structure, and the brown colour appears. At the posterior end a



Text-fig. 3. A horizontal section through the spermatophore ampulla. *d*, the dark-brown colour extending along the inner surface of the inner layer; *a*, a diagrammatic representation showing the arrangement of the spermatozoa.

minute dark-brown slit traverses a small projection of this layer into the papilla. Removal of the papilla, thus exposing the slit, shows that the opening must be blocked, and the contents of the ampulla and papilla absolutely isolated.

4. The inner membrane is a very thin transparent structure lining most of the inner layer. It takes origin from the posterior end of the inner layer, passing in between two adjacent symmetrical thick bodies (the pressure body of Regen) and is attached to the inner layer at the other end of the capsule near the origin of the tube. It is also attached to the same layer all down the walls of the ampulla along its sagittal plane, but loose elsewhere. The inner membrane extends through the posterior part of the inner layer forming another sac in the papilla.

The Contents of the Spermatophore Capsule

1. The pressure body is a translucent mass of thick consistency divided up into two symmetrical parts, each surrounded by a separate transparent membrane and enclosed, at the posterior end of the spermatophore capsule, between the thick inner layer and the inner membrane. In cleared preparations and in sections, each half of the pressure body can be differentiated into a smaller inner and a larger outer part. The two parts show different staining properties but both give protein-positive reactions.

2. A granular substance is found underneath the pressure body inside the capsule surrounded by the inner membrane. It is a viscous fluid, and when examined under the microscope in a drop of water its fine granules show Brownian movement. It is termed by Spann (1934) the seminal fluid and she presumes that it has a nutritive function, since it accompanies the sperm in the spermatheca.

3. The sperm mass occupies the remaining part of the capsule. The heads of the spermatozoa are closely packed in 8–10 layers and directed towards the opening of the spermatophore. Their long tails have a silvery shining appearance, and are packed together below the granular substance. The spermatozoa are immobile in the spermatophore.

The papilla (Text-fig. 1). This is a peculiar structure at the posterior end of the ampulla. It contains a small sperm sac formed by the extension of the inner membrane. The sperm sac is supported by a considerable number of thin membranes (Text-fig. 3) that take origin from the inner layer. As has been stated, the contents of the papilla are completely isolated from those of the ampulla and so never have access to the female organs.

The ampulla varies in size from one insect to another, but it seems that every male produces spermatophores of the same size. The ampulla is generally 1·5–1·7 mm. in length, including the papilla, and 1–1·3 mm. wide in the widest part.

The handle (Text-fig. 1). This is a continuation of the outer layer. It is separated from the ampulla by a short neck, and has two lateral extensions each with a pointed process. At the other end there is an invagination which fits tightly over the pointed end of the spermathecal spout of the female. In a spermatophore detached from its mould the invagination is covered by a gelatinous substance (Text-fig. 1*g*). The handle was called by Regen 'the fixing apparatus' because it fixes the spermatophore in the genital opening of the female. The two lateral extensions fit exactly on the base of the ovipositor from the ventral side, and having the end of the spermathecal spout fitted in the invagination the spermatophore is carried safely by the female for a considerable length of time. The dimensions of the handle are about 1·5 mm. in length and 1·25 mm. in width when the two lateral processes are fully extended.

The spermatophore tube (Text-fig. 1). This is an extraordinarily fine capillary tube originating from the inner layer. It extends through the middle of the handle and about 3·65 mm. beyond it. It gradually tapers towards the end where it terminates in an extremely fine-pointed closure. It has a double wall and is dark brown in colour.

It has been presumed by Jensen (1911), Spann (1934), and others that the spermatophores are chitinous structures. This has been found to have no foundation, since a spermatophore dissolves completely in a hot saturated solution of caustic potash. Millon's and the xanthoproteic tests as well as the ninhydrin reaction prove that it is built up of protein. Fat tests give negative results.

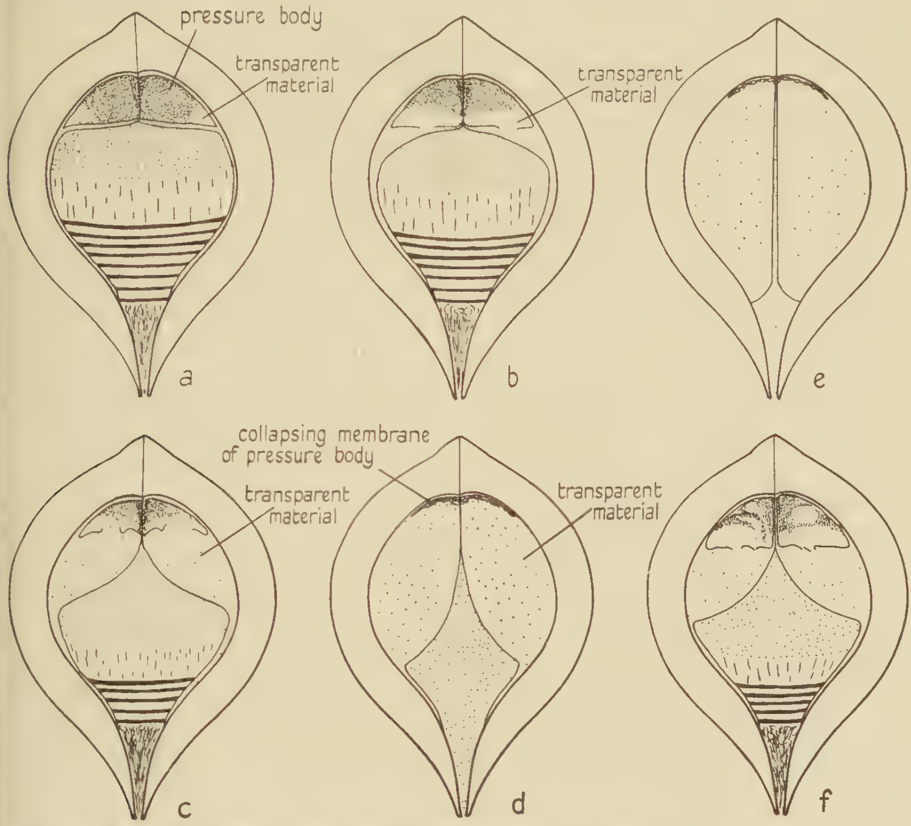
THE PROCESS OF SPERMATOPHORE EVACUATION

The mechanism of sperm transference is in most insects a problem that has not been satisfactorily solved. This is perhaps due to the meagre amount of secretions found in the different parts of the sexual tract, which has made investigation of their chemical composition and physical properties difficult. In the cricket spermatophore the migration of sperm to the spermatheca is carried out mainly if not entirely by forces provided by the ampulla which remains outside the female organs after copulation. The problem is here, therefore, much simpler than it is in those insects, e.g. Lepidoptera, where spermatophores are introduced inside the female bursa, and where sperm, stimulated, activated, and directed by unknown factors, leave the spermatophore and take a most complicated path to the spermatheca.

Regen's explanation. Regen has shown that the fluid enclosed between the outer and the inner layer is of great importance in emptying a spermatophore. As long as the spermatophore is kept in its mould the tube remains closed and the contents of the ampulla have no access to the outside. When the spermatophore was examined in water he could see and remove the closure, so that the sperm started to come out. He presumed that when the spermatophore tube was inserted into the spermathecal duct during copulation the closure would dissolve in the spermathecal duct to allow the sperm to flow out. When the tube was broken on a slide a flow of sperm would also start to come out and would continue for some time, until no fluid remained between the two layers of the ampulla. This also happened when a spermatophore was delivered to a female. When a spermatophore was deprived of this fluid and the tube was broken nothing came out, but when a spermatophore so treated was put in water the flow started. Inside the ampulla, on the other hand, he found the pressure body underwent a swelling process as the spermatophore emptied itself. So a pressure was exerted inside the ampulla, and as the size of the capsule remained constant because of the great resistance of the inner layer all the contents were forced out. The pressure, he deduced, originated when the outer fluid penetrated the inner layer (after the closure at the end of the tube had been removed) and caused the pressure body to swell. He also stated that the pressure body was also pushed to the outside by the pressure it generated on itself.

Description of the process of evacuation. The different stages in the process of emptying a spermatophore were worked out by watching the process under a binocular microscope. When a spermatophore was placed on a slide and had its tube cut somewhere between the handle and the ampulla, the flow of sperm would continue for about 19 minutes. This was followed by the flow of the granular substance, which took about 15 minutes. To make observation easier the ampulla was deprived of the outer layer, which could be easily done when the spermatophore was still in the mould, and dipped in water. When the tube was broken at the above-mentioned point the outflow of sperm started and took about 11 minutes. To cause the process to extend over a long time so that the different stages could be clearly observed, concentrated salt solutions were used instead of water (see Table 1 on p. 289).

Two small transparent areas (Text-fig. 4*a*) first appear on both sides of the ampulla at the periphery of the pressure body. The transparent material increases gradually in quantity inside the two membranes surrounding the two halves of the pressure body; eventually a stage is reached where the two membranes cannot resist the increase in size and burst (Text-fig. 4*b*). The transparent material now occupies the space between the inner layer and the inner membrane on each side of the ampulla. As this material increases in quantity the inner membrane is pushed inwards (Text-fig. 4*c* and *d*) and a pressure is exerted on the contents of the ampulla, causing them to flow out. The contents of the ampulla continue to come out until the translucent pressure body has become entirely transparent. When an intact spermatophore was evacuated on a slide this stage was reached while a certain amount of the granular substance was still in the ampulla (Text-fig. 4*d*). This also occurs with spermatophores delivered to females. In other words complete evacuation is never obtained with spermatophores delivered to females or emptied on a slide. In water or dilute salt solutions, however, the sperm as well as the whole amount of the granular substance come out. In such empty spermatophores the inner membrane, which is at the beginning a smooth stretched structure lining the inner layer, is now folded and takes a vertical position along the sagittal plane of the spermatophore, standing in the ampulla as a double layer partition (Text-fig. 4*e*). In some cases, however, the inner membrane is seen to lose contact with the inner layer along the sagittal line, producing a continuous space surrounding the collapsed inner membrane which is occupied by the transparent material. The two membranes which surround the two halves of the pressure body appear at the end as two collapsed structures at the top of the capsule (Text-fig. 4*d*).



TEXT-FIG. 4*a*, *b*, *c*, and *d*. Four stages in evacuating a spermatophore on a slide by cutting the spermatophore tube somewhere between the ampulla and the handle. These stages represent what happens in the natural condition. *e*, Shows the complete evacuation when stage *d* was put in water. *f*, Shows the result of soaking a spermatophore—which had had the outer layer removed and the evacuating fluid dried up—in 1·5 M sucrose solution for 12 hours. It shows the ampulla as partly evacuated.

In all diagrams the outer layer and the evacuating fluid are not represented.

Role of osmosis. The fact that the evacuating fluid has great importance in emptying the spermatophore and that this fluid diffuses inward when the closure is removed, is true of the spermatophores of both species. A spermatophore that was kept in its mould or on a slide for such a long time in dry air that the evacuating fluid had dried up, was no longer functional; when the tube was broken nothing came out. The outer layer in such spermatophores is folded like that of an empty ampulla. A drop of water, or any dilute salt solution on the ampulla acts like its normal fluid and the sperm starts to come out. The ampulla is normally protected against desiccation by the two muscular flaps almost covering it, which contain a considerable amount of body fluid.

The outer layer, beyond enclosing the evacuating fluid round the ampulla, has no other function so far as the process of emptying the spermatophore is concerned. An ampulla that had this layer removed but still retained traces of the evacuating fluid worked normally when the spermatophore tube was broken. Moreover, an intact ampulla which was left for a while in dry air to get rid of the evacuating fluid still worked normally when dipped in water or any dilute salt solution. The outer layer is, therefore, acting as a permeable membrane, and has no mechanical or physiological effect on the penetration of the evacuating fluid through the inner layer.

Regen stated that when a spermatophore was dipped in water nothing came out unless the closing plug was removed. In the present work, however, in water as well as dilute salt solutions the spermatophore tube sometimes swelled and burst at some points along its length, and so the sperm had access to the outside. In some other cases where this did not occur, and the spontaneous removal of the closure also failed, the thick inner layer cracked and all the contents came out. This shows that water is more efficient in emptying a spermatophore than the natural fluid surrounding the ampulla. This is evident from the fact that the whole amount of sperm comes out in a shorter time when water is used in evacuating the spermatophore than when it is evacuated on a slide (11 against 19 minutes). It follows that the evacuating fluid must have a certain limited osmotic pressure, thereby avoiding rupture of the tube or of the ampulla as long as the spermatophore is kept sealed, as in the mould.

Many spermatophores were deprived of the outer layer of the ampulla, taken away from their moulds and left on a slide for some time to dry. Bits of filter-paper were also used to remove as quickly as possible all traces of the evacuating fluid. When the ampulla became completely dry the spermatophore tube was cut somewhere between the ampulla and the handle, and the ampulla was dipped in a small amount of salt solution in a watch-glass. Every such treated spermatophore was used once only. The results of a typical experiment repeated a number of times are shown in Table 1. No explanation is offered of the curious order of relative effectiveness of the salts, but this order was found to occur in every case.

It is evident, therefore, that with $MgSO_4$, KCl, and KNO_3 2·25 M solutions, or below, execute the emptying process while 2·5 M solutions maintain conditions inside the ampulla unchanged. With $NaNO_3$ and NaCl 1·25 M solutions or below, evacuate the ampulla while 1·5 M solutions just keep conditions unchanged. With sucrose the corresponding concentrations are 1·75 M and 2 M respectively.

Moreover, the degree of evacuation was found to be inversely proportional to the molecular concentration of the solution. Text-fig. 4*f* shows the amount of sperm evacuated from a spermatophore kept in 1·5 M sugar solution overnight. In such a solution complete evacuation was never obtained as an equilibrium between the two solutions on both sides of the inner layer was reached.

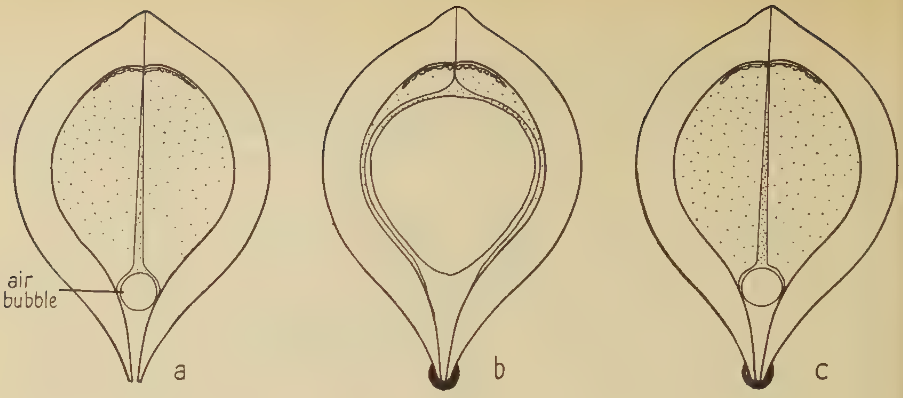
TABLE 1. *The Effect of Different Salt and Sugar Solutions on the Evacuation of Spermatophores*

Salt or Sugar	*Molecular concentrations*						
	1·0	*1·25*	*1·5*	*1·75*	*2·0*	*2·25*	*2·5*
$MgSO_4$	+*n*	+	+	+	+*s*	+*v.s*	—
KCl	+*n*	+	+	+	+*s*	+*v.s*	—
KNO_3	+*n*	+	+	+	+*s*	+*v.s*	—
$NaNO_3$	+*s*	+*v.s*	—	. .	. .	. .	. .
NaCl	+*s*	+*v.s*	—	. .	. .	. .	. .
Sucrose	+*n*	+	+*s*	+*v.s*	—	. .	. .

+, contents coming out; -, nothing comes out; *n*, flow is normal as compared with water; *s*, flow is slow; *v.s*, flow is very slow.

By using solutions of the same salts but of higher concentrations, and by introducing an air bubble the inner membrane can be forced to take its original position. A spermatophore was first deprived of the outer layer, taken out of the mould, had the tube cut somewhere between the handle and the ampulla and dipped in water; this produced quick and complete evacuation. When the ampulla became totally empty it was taken out, placed on a slide and left for a while. The ampulla eventually lost some of its water, allowing an air bubble to get between the two halves of the collapsed inner membrane (Text-fig. 5*a*). The open end was now sealed with paraffin wax, and the ampulla was dipped in any of the more highly concentrated solutions, e.g. 2·75 M of the first group or 1·75 M of the second group. The air bubble expanded and the inner membrane moved outwards and eventually regained approximately its original position (Text-fig. 5*b*). This took about 10 minutes. The material enclosed between it and the inner layer maintained, however, its transparent appearance. If the solution was replaced by water or any dilute solution the inner membrane was forced again to collapse and the air bubble to take its small original size (Text-fig. 5*c*). The whole process was repeated several times as the blocked empty ampulla was transferred from one solution to the other. Without an air bubble it was hardly possible to detect any movement of the inner membrane.

The experiment was repeated with an intact spermatophore evacuated by cutting the tube on a slide (Text-fig. 6). As has been mentioned above, complete emptying can never be obtained in this way. The remaining part of the granular substance was found to hinder the entrance of an air bubble. This difficulty was overcome by blocking the opening of the ampulla (Text-fig. 6*a*) as the flow stopped, and dipping it in a concentrated solution, e.g. 2·75 M of the first group. After a while the ampulla was taken out and the plug removed, when an air bubble immediately entered (Text-fig. 6*b*). The opening



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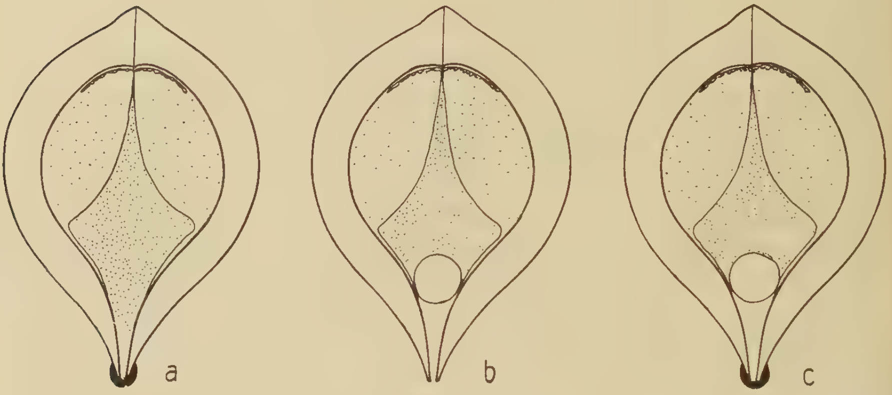

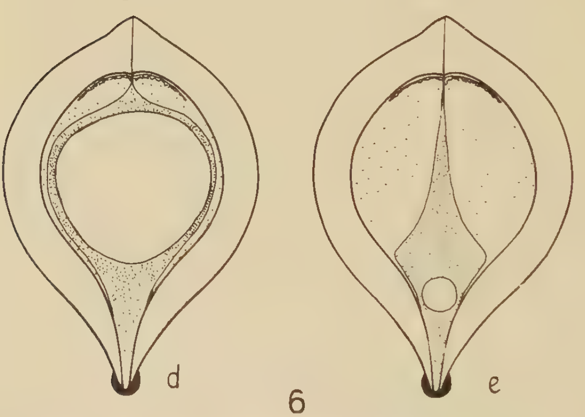

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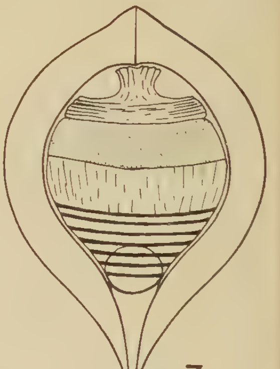

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Text-fig. 5*a*. Represents a spermatophore ampulla that had had the outer layer removed, the evacuating fluid dried up and was emptied in water. An air bubble entered as the ampulla was left to dry. *b*, Shows the expansion of the air bubble when the ampulla was sealed and placed in a 2·75 M $MgSO_4$ solution. The inner membrane was forced to take approximately its original position. *c*, The air bubble resumed its original size as the ampulla was immersed again in water.

Text-fig. 6*a*. Represents a spermatophore ampulla that has been evacuated on a slide by cutting the spermatophore tube somewhere between the ampulla and the handle. It was sealed and placed for a short time in a 2·75 M $MgSO_4$ solution. *b*, The seal was removed and an air bubble entered. *c*, It was sealed again. *d*, The air bubble expanded as the ampulla was placed in the 2·75 M $MgSO_4$ solution. *e*, The ampulla was returned to water. The inner membrane took nearly the position shown in *a*, and the air bubble was forced to take a smaller size.

Text-fig. 7. Represents a spermatophore ampulla that had had the outer layer removed, the evacuating fluid dried up and placed in a 2·25 M sugar solution for 12 hours. The pressure body shrank and an air bubble entered as the ampulla was taken out.

was now sealed again and the ampulla placed in the solution. The same processes of expansion and contraction occurred when the ampulla was transferred from one solution to the other (Text-fig. 6*c*, *d*, and *e*).

A spermatophore that had had the outer layer removed and the fluid dried up could be preserved in any of the solutions which keep conditions inside the ampulla unchanged for a long time—a period of 2 weeks was tried—and still worked normally when water or any dilute solution was used. With regard to temperature, Regen found that spermatophores did not work when examined at low temperatures which he presumed would be near the freezing-point of the pressure body.

It is clear that the whole process is carried out by osmosis. The inner membrane moves inwards as water diffuses in and so exerts a pressure on the contents of the capsule, and moves outwards as water diffuses out. The inner layer functions, therefore, as a semi-permeable membrane separating two fluids of different osmotic pressure, the evacuating fluid and the pressure body. The difference between the osmotic pressure of the two fluids is very great. The depression of the freezing-point (Δ) was determined. Four specimens were taken of each and the result was as follows:

The evacuating fluid, $\Delta = 0{\cdot}898°$ C
The pressure body, $\Delta = 3{\cdot}062°$ C.

Water can diffuse through any part of the inner layer. This has been proved by covering its surface gradually with wax. Any uncovered area at any point on the surface, however small, will allow water to diffuse in or out. As a control ampullae that were covered totally with wax did not work.

MIGRATION OF SPERM TO THE SPERMATHECA

It remained to find out whether the sperm would migrate from the ampulla to the spermatheca if the spermatophore was deprived of the evacuating fluid. Sexually excited males were chosen and the outer layer of the spermatophore ampulla was removed and the fluid dried up. When the males had recovered from anaesthesia they were provided with virgin females. This was carried out in the hope that such treated spermatophores could be delivered to the females, but they were always pushed out and dropped by the formation of fresh ones. So the same operation was carried out immediately after the spermatophore was delivered. In order to prevent the females eating the spermatophores their heads were amputated and the cuts were sealed with wax. Four virgin females were treated in this way, and their spermathecae as well as the spermatophores were examined at intervals of ½, 1, 6, and 12 hours after copulation. In every case almost the same amount of sperm was found in the spermatheca, but the delivery of the whole amount was never obtained. On the other hand it was found that in an hour the whole amount of sperm and most of the seminal fluid would migrate from an intact spermatophore to the spermatheca. So it is evident that in the four treated spermatophores certain amounts of sperm had migrated during the operation and drying up of the evacuating fluid. When the ampulla became completely dry the flow of sperm almost certainly came to an end. Once the sperm reach the tip of the spermatophore tube they become very active, stimulated most probably by the spermathecal fluid, and carry on the remaining part of the way to the spermatheca apparently by their own movements. Sperm obtained from the spermatheca were more active in Ringer than those obtained from the spermatophore or the vesicula seminalis.

ACKNOWLEDGEMENTS

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SUMMARY

The house cricket spermatophore is largely protein and does not contain any chitin. It is composed of an ampulla, a handle, and a long capillary tube terminating in a pointed closure.

The wall of the ampulla comprises four different layers: an outer thin layer, an evacuating fluid, a thick strong inner layer, and a transparent inner membrane. In between the inner layer and the inner membrane, at the top of the cavity, there is the pressure body, a protein mass, divided into two halves. Each half is surrounded by a thin membrane.

The process of emptying a spermatophore is due to osmosis. It has been shown experimentally that the strong inner layer of the ampulla acts as a semi-permeable membrane separating two fluids of different osmotic pressure —the evacuating fluid and the pressure body. When the closure at the tip of the tube is removed, the pressure body swells, exerting a pressure on the sperm as the evacuating fluid passes inwards.

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Further Remarks on the Golgi Element

BY

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(*From the Department of Zoology and Comparative Anatomy, Oxford*)

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INTRODUCTION

IN 1944 I published a long paper on the structure and chemical composition of the Golgi element. Since then the subject has been actively pursued in the Department of Zoology and Comparative Anatomy at Oxford by Dr. O. L. Thomas, Dr. A. J. Cain, Dr. Su-Hsuen Wu, and myself. In 1948 Dr. G. C. Hirsch visited us for discussion and practical collaboration. The main purpose of the present paper is to consider whether the view of the Golgi apparatus presented in the earlier paper is correct, and if not, how it should be changed. The opportunity is also taken to give the details of improved technique.

It is thought convenient to start by giving a general account of the Golgi element as it appears to me in the light of our researches, and by explaining the nomenclature that will be used. This will considerably simplify the exposition of the new results. The latter were obtained by a re-examination of the same kinds of cells as formed the basis of the 1944 paper. Some investigations of another kind of cell are mentioned on pp. 305–6.

THE STRUCTURAL PLAN AND NOMENCLATURE OF THE GOLGI ELEMENT

The name 'Golgi apparatus' applies strictly to a network of particles of silver seen in the cytoplasm of the Purkinje cells of the cerebellum of the barn-owl *Tyto alba* (called by Golgi (1898) *Strix flammea*), and of certain other nerve-cells, when particular reagents have been applied in a particular sequence. There is no evidence that any single constituent corresponding at all closely with this network exists in the living cell. Dr. Thomas's investigations (1948) suggest that it represents the Golgi bodies, in the

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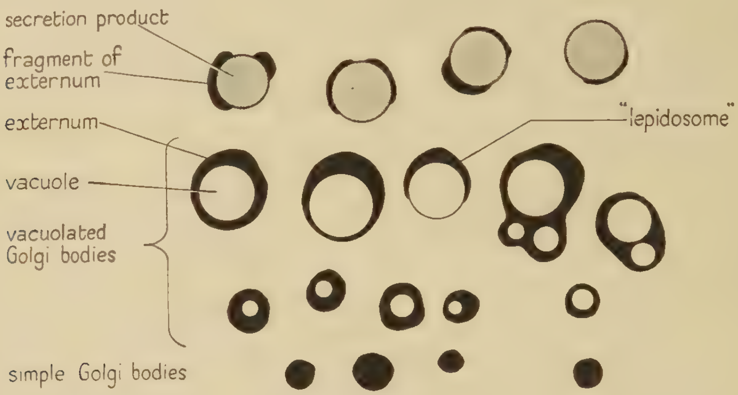
restricted sense in which this expression will be used in the present paper, together with mitochondria and artifact (that is, particles of silver deposited at random on the surface of other particles already deposited). It is well known that the classical methods for the 'Golgi apparatus' often impregnate mitochondria. Rámon-Cajal himself freely allowed this, and illustrated mitochondria in the pancreas and submaxillary gland, beautifully impregnated by his uranoformol method (1933, Figs. 3 and 457).

This being so, one is tempted to discard altogether the name of Golgi when referring to the objects described in this paper. However, they must have a distinguishing name, and it is true that in many cells the method of Golgi and the other classical methods do impregnate the region of the cell in which the Golgi bodies *sensu stricto* lie, more readily than they impregnate other cellular constituents (though they do not reveal the true structure of this region). It seems inevitable, therefore, that for the present, at any rate, the name of Golgi must be retained.



TEXT-FIG. 1. Diagram illustrating the author's opinion as to the structural plan of the fully developed Golgi element, as seen in a very thin section.

In what follows, the nomenclature adopted will be the simplest, most descriptive, and least tendentious that I can devise.

The word 'apparatus' has little to commend it. When one wishes to deal in the most general way with characteristic cellular constituents, it is legitimate to speak of nuclear, mitochondrial, and Golgi *elements*. The *Golgi element*, then, consists of separate *bodies*, which are rounded, but seldom perfectly spherical (see Text-fig. 1). There are usually many Golgi bodies in one cell. The Golgi body may be *simple*, i.e. apparently homogeneous, or it may be *vacuolated*, in which case it consists of an *externum* investing a *vacuole*. The lipoid nature of the simple Golgi body and of the externum is revealed especially by its affinity for sudan black. When a series of stages in time can be observed, it is found that simple Golgi bodies occur first and vacuolated ones later. The latter almost certainly arise by vacuolation of simple bodies. The externum seldom covers the whole vacuole in a layer of even thickness:

the degree of its eccentricity in relation to the vacuole varies considerably in different cases, and sometimes it appears to be reduced to a small cap or band, though a very thin layer may perhaps even then extend over the whole vacuole. The vacuole is perfectly spherical; this fact, coupled with the impossibility of demonstrating any solidity in it, suggests its watery nature, which is the cause of its disappearance in routine microscopical preparations even when care is taken to preserve the lipoid that surrounds it; the lipoid is pulled about by protein precipitation in its vicinity and does not retain its shape exactly, and there is then nothing to suggest the previous existence of the vacuole. The vacuole has no affinity for sudan black.

One, two, or several vacuoles may be present in one body, which may thus be *uni-*, *bi-*, or *multivacuolate.*

In large vacuoles new substances, the *secretion-product*, may begin to appear. Very diverse substances appear in this way in the Golgi bodies of different cells, and no generalization can be made about them except that they are of protein or lipoid nature. When the secretion product has been formed, the material of the lipoid sheath does not form a complete covering. Eventually the secretion-product becomes free from any investment and no longer forms part of the Golgi element.

The simple Golgi body corresponds to the 'Praesubstanz' of my friend, Dr. G. C. Hirsch (1939), and the vacuolated body to his 'System'. My reasons for using a different terminology are these. The word Praesubstanz is not really applicable to a separate *object*, but only to the substance of which that object is made; and the word System is not purely descriptive, but involves theoretical considerations. The vacuole corresponds to the 'Internum' of several authors, but Dujardin's word (1835) is applicable here and is more descriptive.

The view of the Golgi apparatus just presented agrees in general with that put forward in my 1944 paper, but differs in the following points:

1. I no longer call the vacuole the 'neutral red vacuole'. It is true that it usually has a particular affinity for neutral red *intra vitam*, but as this is not invariably so, it seems better to discard the name.
2. I no longer place a diffuse lipoid-containing substance between the vacuoles, because it cannot be seen in life and there are undoubtedly many cases in which it cannot be demonstrated by any means; and even when it seems to occur in fixed preparations, one may be deceived by the out-of-focus appearance of sudanophil material lying above or below. This is not a matter on which I would wish to dogmatize, but I no longer regard the diffuse lipoid as a necessary constituent.

METHODS

Only the methods of general application are considered under this head. Special techniques, appropriate to particular tissues, are given in the descriptive part of the paper.

Vital Methods

In all the researches of my associates and myself, chief reliance has been placed on the study of the living cell. In this field the chief advance has been the use of phase-contrast microscopy. The method of Kempson, Thomas, and Baker (1948) has been adopted, with certain improvements that will be published shortly. Dark-contrast ('positive' phase-contrast) has on the whole been found preferable to bright in these studies.

Sodium chloride solution of the proper osmotic pressure, with the addition to each 100 c.c. of 0·2 c.c. of 10 per cent. calcium chloride solution, remains the standard medium for the examination of living cells. I have tried more complex solutions without noticing the least difference in the structure of the Golgi bodies, with the possible exception that Hédon-Fleig saline may be preferable for the cells of the snail.

Dahlia and neutral red (chloride) remain the most useful vital dyes. These are kept in solutions at 0·5 per cent. in distilled water. When required, 0·1 c.c. of the dye solution is added to 5 c.c. of the saline solution just described, and the cells are teased or otherwise immersed in this. Stronger and weaker solutions are used for particular purposes.

The Improved Sudan black Technique

Sudan black was introduced into biological technique by Lison and Dagnelie (1935). It was first used to show the Golgi bodies by myself (1944) and it has been adopted by my associates. Foster (1947) has used it in a study of the pituitary gland.

The design of the sudan black technique for the Golgi element rested on three principles: (1) that no protein precipitant should reach the cells until fixation was complete; (2) that the colouring agent should act simply by solution in the substance coloured; and (3) that the technique should present a picture of the Golgi bodies resembling that seen in living cells, so that it might be used with some confidence on cells that cannot be isolated for examination in the living state. I have now considerably improved the technique, while adhering to these principles. The new technique bears a resemblance to that of Ciaccio (1910).

The intention has been to reduce the tendency of sudan black to colour mitochondria and ground-cytoplasm, while increasing its tendency to colour the simple Golgi bodies and externa. The new technique depends upon the fact that treatment of the tissue in bulk with potassium dichromate greatly facilitates the subsequent colouring of these cellular constituents with sudan black, as I found some years ago (see Cain, 1947, p. 152), while formaldehyde antagonizes the subsequent colouring of the mitochondria and cytoplasm by sudan black. The technique is designed in such a way that the actions of potassium dichromate and formaldehyde are balanced against one another. In a successful preparation simple Golgi bodies and externa are dark blue or blue-black, while the vacuole is colourless and the cytoplasm either colourless or nearly so.

The following solutions, &c., are required:

Formaldehyde-saline

Formalin	10 c.c.
Sodium chloride, 10 per cent. aq.	7 c.c.
Distilled water	83 c.c.

Keep marble chips in the solution.

Dichromate-formaldehyde

Potassium dichromate, 2.5 per cent. aq.	88 c.c.
Sodium chloride, 10 per cent. aq.	7 c.c.

To 19 c.c. of this stock solution add 1 c.c. of neutral formalin. (Keep marble chips in the formalin bottle to maintain neutrality.)

Potassium dichromate, 5 per cent. aq.

Gelatine for embedding

Soak 25 gm. of powdered gelatine for an hour in 100 c.c. of a 0·2 per cent. aqueous solution of sodium *p*-hydroxybenzoate or Moldex. (The latter is obtainable from R. Campbell, 7 Idol Lane, London, E.C. 3.) Then warm in an incubator maintained at 37° C. till the gelatine has dissolved; filter through muslin while still warm. (The sodium *p*-hydroxybenzoate or Moldex prevents the growth of moulds and bacteria. It is superior to cresol in preventing the growth of the bacterium that liquefies gelatine gels.)

Formalum

Formalin	20 c.c.
Potassium alum, 5 per cent. aq.	80 c.c.

Keep marble chips in the solution. This new agent for hardening gelatine will be found useful by workers with the freezing microtome. Both gelatine blocks and sections may be preserved indefinitely in it. It facilitates the cutting of thin sections, as it makes the gelatine very hard; and the sections are not sticky. (Formalum must not be used in the acid haematein test for phospholipines (Baker, 1946), as the alum would react with the haematein.)

Sudan black

A saturated solution in 70 per cent. alcohol. It suffices to allow 0·5 gm. to 100 c.c. It is essential that the solution should be thoroughly saturated. If a solution is required quickly, boil for 10 minutes with reflux condenser.

The following specimens of this colouring agent are good:

Sudan black 404194/480705 of the British Drug Houses;
Sudan black No. 2 of E. Gurr;
Sudan black 349 of W. T. Gurr;
Sudanschwarz B N of Höchst.

Some of the specimens of sudan black on the market are useless for this technique.

Mayer's Carmalum (Mayer, 1892).

Farrants's medium (can be obtained ready-made from G. T. Gurr).

I. Fix a piece of tissue not more than 3 mm. thick in formaldehyde-saline for 1 hour.
II. Transfer without washing to dichromate-formaldehyde and leave for 5 hours.
III. Transfer without washing to 5 per cent. aqueous potassium dichromate and leave overnight (about 18 hours) in this solution at room temperature.
IV. Leaving the tissue in the same solution, transfer to the paraffin oven at 60° C. and let it remain there for 24 hours.
V. Wash for 6 hours in running water.
VI. Leave overnight in the prepared gelatine, melted at 37° C.
VII. Cool the gelatine (preferably in a refrigerator), cut out a rectangular block containing the specimen, and place it in formalum. Place a marble chip in the capsule or tube. Leave overnight (or any convenient longer period).
VIII. Cut sections at 8 or 10μ on the freezing microtome.
IX. Transfer a section to 70 per cent. alcohol. It is best to transfer sections from fluid to fluid, up to stage XV, in a porcelain pot with small holes in the bottom (e.g. the Royal Worcester porcelain thimble No. a.4756, Size 2, obtainable from Messrs. A. Gallenkamp).
X. Transfer to the sudan black solution and leave for $\frac{1}{2}$–4 minutes. (The best period is usually about 2$\frac{1}{2}$ minutes.)
XI. Wash in 70 per cent. alcohol for 5 seconds.
XII. Wash in 50 per cent. alcohol for 1 minute.
XIII. Wash in water. Sink the section if it floats.
XIV. Transfer to Mayer's carmalum and leave for 2–4 minutes. (The best period is usually about 3 minutes.)
XV. Rinse in distilled water. (A precipitate may form if tap-water is used at this stage.)
XVI. Remove the section from the porcelain pot and wash for about 2 minutes (or any convenient longer period) in a tongue-jar or other fairly large bowl of water (tap or distilled: it makes no difference).
XVII. Wash again in a large bowl of water.
XVIII. Transfer to a Petri dish of water. Float the section on to a glass microscopical slide. Remove the slide from the water.
XIX. Mount in Farrants's medium.
XX. Attach a clip to hold the cover-glass to the slide. Leave overnight in the paraffin oven, to harden the mounting medium. (One may examine the slide after a quarter of an hour and then return it to the oven to complete the hardening.)

Result. If successful, the technique gives this result: simple Golgi bodies and externa, dark blue or blue-black; Golgi vacuoles, colourless; cytoplasm, colourless or pale grey-blue; chromatin, pink or red.

Never attempt to judge the colouring until the section is mounted. An immersion-objective must then be used. If the result is not good, try another section, using different periods in the colouring agents.

The only difficulty in this technique is caused by the fact that the sudan black solution must be saturated. As a result, the slightest evaporation causes a precipitate; this, if it occurs, will be found on the surfaces of the section, or occasionally, in loose tissues, between the cells: it never occurs within cells. To minimize this trouble, be careful to keep the sudan black solution in a stoppered jar while the section is being coloured.

If there is difficulty in colouring the Golgi bodies, it is worth while to repeat the whole process with the omission of stage I; that is, fix the tissue directly in dichromate-formaldehyde.

No claim is made that simple Golgi bodies and externa are in every case exhibited by the sudan black technique, nor that a dark blue or blue-black coloration necessarily indicates a Golgi body: for instance, triglycerides react in the same way. It is useful to fix a piece of tissue in formaldehyde-saline and colour a section with sudan IV; this will show triglycerides and certain other lipoids, but not Golgi bodies. When triglycerides are very abundant in a cell, they interfere with the technique.

All that is claimed for the method is that when it is used on kinds of cells that can also be examined alive, it gives pictures that resemble the living condition fairly closely; that it often shows Golgi bodies and externa very well; and that it is not 'capricious' nor 'patchy' in its effects, as the classical methods for the Golgi element are, for it can be relied on to give the same appearance every time.

I hope that anyone who decides to try this technique will learn it upon an 'easy' tissue. I particularly recommend the intestine of the mouse: it is scarcely possible to fail with this. Cut out a piece of empty intestine about a centimetre long and drop it into the formaldehyde-saline. After 5 minutes (when the outer layers have hardened and the tissue is less likely to lose its form), open it by a longitudinal cut from one end to the other, taking great care not to do any unnecessary damage to the villi. Leave the section only 1 minute in sudan black and $2\frac{1}{2}$ minutes in carmalum. The resulting preparation should show the Golgi bodies clearly in every cell of the epithelium and of the glands of Lieberkühn.

Those who try this technique may like to try also that of Thomas (1948), who colours the Golgi bodies with sudan black in paraffin sections.

RESULTS

The Epithelial Cell of the Intestine of the Mouse

It is much easier to display the epithelial cell of the intestine of a mammal for *intra vitam* study, than the corresponding cell of the newt; and I have therefore changed to the study of the former.

To display this cell in the unfed mouse, proceed as follows: Choose a part of the intestine that is free from food and cut out a piece about a centimetre long. Place it on a glass plate and open it by a longitudinal cut with scissors; lay it flat, with the inner side uppermost. Keep it moist with a few drops of the saline solution (p. 296). Holding one end of it still, scrape it several times with a sharp straight scalpel; the villi will be cut off and will accumulate near the edge of the scalpel. Wash them with saline on to a microscopical glass slide; cover; ring the coverslip with vaseline.

The villi will be seen under the low power of the microscope as objects resembling in shape the last joint of a finger. Place the middle of a villus in the optical axis and adjust for phase-contrast microscopy with the oil-immersion $\frac{1}{12}$ in. or $\frac{1}{14}$ in. objective, using dark contrast ('positive' phase-contrast).

The free border of the cell lies towards you. Focusing up from near the middle of the villus, you will see first the basal group of mitochondria, then the nucleus, then the Golgi bodies, then the apical group of mitochondria (these are the hardest to see), and finally the intercellular band ('Kittleiste') and free border. *The Golgi bodies are the most evident objects in the cell.* They are situated where the classical methods show a network, but have quite a different structure.

The bodies are of various sizes, the largest being about 2μ in diameter. The largest contain more than one vacuole, while the small ones have a single vacuole or none. The material of the simple Golgi bodies and of the lipoid sheaths appear dark by 'positive' phase-contrast microscopy. It is not necessary to show a figure of these bodies, for they are sufficiently well represented by the univacuolate body on the left of Text-fig. 1 and the bi- and multivacuolate ones on the right.

Nothing is seen resembling a fully formed secretion-product.

The same appearance is seen if the villi, after being cut off, are soaked for 15 minutes in one of the vital dyes (p. 296) in a watch-glass, with occasional stirring, and then examined by ordinary microscopy. Both dahlia and neutral red colour the Golgi bodies. The exact distribution of the dyes is difficult to determine, but the dahlia certainly stains the externum blue. In the vacuolated bodies neutral red colours the vacuole, but I cannot state with confidence that it does not also stain the externum.

It may be recalled that in the newt, dahlia stains the externum blue and the vacuole yellow or red; neutral red colours the vacuole only (Baker, 1944).

The sudan black technique shows the Golgi bodies of the intestinal epithelial cell of the mouse very much as they appear in life. The sudan black colours only the lipoid material, leaving the vacuoles colourless. The only obvious difference from the living state is that the bodies are nearer together (presumably on account of the contraction of the cytoplasm). The vacuoles are sometimes rather difficult to see.

The Primary Spermatocyte of the Common Snail, Helix aspersa.

In the very young primary spermatocyte, in which there is as yet very little cytoplasm, there are only a few small simple Golgi bodies, uniformly coloured by sudan black.

It is necessary to correct in several respects the account of the Golgi bodies in the large primary spermatocyte given in my 1944 paper. Dr. Wu and I find that neutral red, especially when used at higher concentrations than that suggested on p. 296, gives rise to pink or red vacuoles where none existed before. These vacuoles usually arise in the Golgi region, sometimes in contact with the 'lepidosomes'; but they sometimes arise in the Golgi region not in contact with any lepidosome, and, when high concentrations of the dye are used, they may be seen to form in other parts of the cytoplasm and to grow gradually to a considerable size. It is therefore clear that judgements as to the structure of Golgi bodies should not be made until a cell has been carefully studied in life, without the use of neutral red. It must, however, be mentioned that the production of artificial vacuoles by neutral red does not occur in most cells: the spermatocyte of the snail is exceptional in this respect.

At first sight there appears to be nothing exactly corresponding to the typical Golgi body in a large primary spermatocyte. Instead one sees the 'banana-shaped' lepidosomes, dictyosomes, or batonettes. The name *lépidosomes* was given by Parat (1926) from their resemblance to *écailles*, and he and Painlevé (1926) remark that these bodies are often *squameux*; but he nowhere gives a careful description or figure that really substantiates these words. The lepidosomes, which are easily seen without staining, are readily coloured by dahlia and many other basic dyes *intra vitam* (never by neutral red), and by sudan black in fixed preparations. When they are attentively studied, one notices that it is very difficult to form a true opinion of their shape. As one focuses the microscope up and down with a delicate fine adjustment, the image of the lepidosome often moves a considerable distance, though the cell remains perfectly still. The banana-shaped appearance is a view in optical section and does not properly represent the whole object, which must be to some extent spread out like a scale. At my request, Dr. A. J. Cain has carefully studied the apparent movement of the lepidosome when one focuses up and down, and he confirms that the appearance can only be explained by the hypothesis that the object is commonly scale-like, and not shaped like a banana or rod. Indeed, Parat's name is a good one, though he did not himself fully explain his reasons for choosing it.

In an unstained living cell the lepidosomes appear grey. It seems certain that this is because they are of higher refractive index than the surrounding medium, and so shaped as to refract some of the light coming from the condenser of the microscope in such a way that it does not enter the objective. A bright line is usually seen on the concave side of the lepidosome. It is not necessary to suppose that this represents another constituent of the object, though Dr. Wu was inclined to think so; rather it represents a region of the lepidosome that acts to some extent as a lens in converging light into the objective. Such alternations of dark and light must occur whenever an object with curved outlines is made of a material of higher refractive index than its surroundings. The bright lines move with the dark when the focus of the microscope is changed. The fact that the lepidosome has a high refractive index is shown by its appearing dark when examined by 'positive' phase-contrast; further, its affinity for sudan black shows it to contain or consist of lipoid. The bright line is not seen when phase-contrast is used.

If one examines a medium-sized lepidosome with the utmost care, focusing continually up and down, one reaches the conclusion that it is *a mass of matter applied to parts of the surface of an invisible sphere*: I can think of no other description that is adequate. When phase-contrast is used, the sphere can sometimes (but by no means always) be seen. I have shown it to Mr. P. C. J. Brunet and Dr. A. J. Cain, who allow me to say that they have seen it with perfect clarity; they have both drawn the object as a sphere with a dark mass spread over part of the surface. Our studies strongly suggest that this structure exists, or is readily assumed, during the latter part of the meiotic prophase. It was, indeed, seen long ago by Perroncito (1910) in *Vivipara vivipara*, especially when the primary spermatocyte was actually dividing (see his Fig. 31 on Plate 1); but he made the mistake of supposing that he was seeing rings or disks.

Exceptionally large lepidosomes seem not to fall within these generalizations: parts of them may perhaps hang free, not applied to the surface of a vacuole. They are too infrequent, however, to be subjected to detailed study by different methods.

The sudan black technique was used for the study of the primary spermatocyte in sections. Great care must be taken in dissecting out the ovotestis; otherwise some of the nurse-cells will be damaged and their lipoid droplets, coloured by sudan black, will lie about in the alveoli and tend to obscure the spermatocytes. The period of colouring in sudan black should be reduced to 1½ minute.

In sections coloured with sudan black the Golgi body is not so difficult to interpret as in the living state, for the uncoloured vacuoles are clearly seen, with their lipoid sheaths. It would appear that fixation has somehow spread the lepidosome farther over the surface of the vacuole, and thus made the whole appearance much more similar to that seen in life in other kinds of cells; the very sharp distinction between the material of the lepidosome and the white of the vacuole helps to make everything clear.

If this interpretation is correct, the lepidosome is an externum of a special kind: it is spread on rather a small part of the surface of a vacuole that is difficult to see in life and does not stain with neutral red. (See the diagram of a lepidosome in Text-fig. 1.)

It is to be observed that this view of the Golgi apparatus of the primary spermatocyte of the snail accords fairly closely with that of Monné (1938, 1939). The main difference is that he considered the vacuole to be of the shape of a biconvex lens, not a sphere. He regarded the lepidosome as spread on its surface, being particularly thick round the rim of the lens; and he showed that when the living cell is treated with ammonium chloride solution, the material constituting the lepidosome distributes itself nearly evenly over the vacuole, so that an appearance resembling the familiar type of Golgi body is produced.

In the shortlived secondary spermatocyte the form of the Golgi bodies resembles that seen in the primary. In the spermatid the Golgi bodies clump together at first and then often separate while taking up their position well behind the nucleus; the very small acrosome meanwhile appears at the front end of the spermatid, without any apparent connexion with the Golgi bodies. We are not able to offer any suggestion as to the function of the Golgi bodies in this particular spermatogenesis. It is well known that in very many cases they migrate to behind the nucleus after secreting the acrosome, and some of them remain there while others are thrown off with the residual cytoplasm. The evidence from *Helix* suggests that the Golgi bodies may play a part in the development of the region of the spermatozoon posterior to the nucleus; for it is not likely that such a large cellular constituent should be functionless. This suggests that in the spermatogenesis of most animals the Golgi bodies may have another role besides that of secreting the acrosome.

Dr. Wu and I devoted a considerable amount of time to an attempt to learn something of the structure of the Golgi bodies in primary spermatocytes by varying the pH and osmotic pressure of our saline solutions. Unfortunately we were not able to obtain any information in this way. We found the Golgi bodies remarkably resistant, maintaining their form even when the cytoplasm was greatly shrunken or swollen. Monné (1938) had already demonstrated their resistance to hypertonic solutions in the case of this particular cell.

The Nerve-cell

In my 1944 paper I put forward the opinion that in the cells of the anterior mesenteric ganglion of the rabbit the Golgi bodies are represented by subspherical objects, each consisting of one or more vacuoles surrounded by a sudanophil externum. This opinion was in sharp opposition to the generally accepted opinion as to the structure of the Golgi element in nerve-cells. It was generally supposed that the true structure was a network.

Dr. O. L. Thomas devoted his two years of study at Oxford to the cytoplasm of nerve-cells. I followed in detail every stage in his work; but as I have not pursued the investigation of the nerve-cell in my own researches, this section of the paper will be short. The reader is referred to Dr. Thomas's papers (1947, 1948). He worked with both living and fixed cells. In his study of the nerve-cell of the anterior mesenteric ganglion of the mouse he confirmed my findings on the rabbit in every respect, except that he saw no special region of diffuse lipoid in the cell; and, as has been remarked above, I no longer regard this as a necessary constituent of the Golgi element. In his general study of the cytoplasm of nerve-cells, however, he went very much farther than I did. He showed how a secretion-product appears within the vacuole, as though the cell were a gland-cell. He also made a careful study of the gradual deposition of osmium hydroxide in the cell in the Mann-Kopsch technique. He showed how the externa are blackened first, then the interiors of the Golgi bodies, and then the mitochondria; finally a non-specific deposition occurs, which thickens the already blackened objects and joins them together into the well-known net.

Cain (1948) confirmed Thomas's work, so far as it referred to neurones of *Helix*, and extended it to other genera of pulmonate gastropods. He found that the orange substance ('lipochrome' of previous authors), which accumulates in the vacuoles of the Golgi bodies of these animals, is carotenoid.

Remarks on Chemical Composition

In my 1944 paper I brought forward strong evidence from various sources, not amounting to histochemical proof, that the lepidosomes of the spermatocytes and spermatids of the snail consist of or contain lipine. In a review of the subject I showed that up to that time no real evidence had been produced as to their chemical composition, beyond the fact that they consisted of or contained a lipoid, and even this knowledge rested not on secure histochemical foundations, but mainly on the observation that they are not usually seen after routine histological treatment involving the use of lipoid-solvents. The use of sudan black demonstrates the lipoid nature of simple Golgi bodies and externa. Dr. A. J. Cain (1947), by employing the acid haematein test (Baker, 1946, 1947), was the first to obtain further information by means of strict histochemistry. He demonstrated the presence of phospholipine in the Golgi lipoid of the epithelium of the alimentary canal of the leech *Glossiphonia*. Phospholipine has been detected in the same way in the Golgi bodies of the nerve-cell of *Helix* by Thomas (1947) and Cain (1948), and in sebaceous cells of man by Montagna *et al.* (1948).

It is noteworthy that in the majority of cases the acid haematein test gives a negative result with Golgi bodies, while mitochondria react positively. It seems certain that there is some other lipoid constituent beyond phospholipine, and it is a matter of great importance to discover what this constituent is. We only cover up our ignorance by speaking of 'Golgi material'. What is wanted is knowledge expressed in terms that mean something to a chemist.

DISCUSSION

There is no cellular constituent that is regarded with such a widespread feeling of uncertainty as the Golgi element. The reason for this is evident enough. The classical methods used in its investigation are no longer of much value except for directing attention to a particular part of a cell: they are neither morphological nor histochemical. Scarcely any attempt has been made to show that they reveal, in its natural form, something already existing in the cell during life. Instead, there has been a tendency to work towards a desired end: if the final preparation shows a desired picture, it is 'good'. The methods give no chemical information except the fact that, under certain arbitrarily chosen conditions, silver nitrate or osmium tetroxide is reduced. This, in itself, does not permit any valid deduction to be drawn as to the chemical composition of the objects blackened. Work of this calibre is not recognized in any other branch of science, and it is natural that there has been a strong reaction against it, especially in America. There has been a feeling that the subject is unworthy of serious consideration.

Parat (1928) was the first to undertake a comprehensive scientific investigation of the subject, by directing his microscope towards that part of the living cell in which the classical methods reveal the 'apparatus'. His great contribution was the finding that vacuoles can generally be discovered in this region. He called them the neutral red vacuoles because they usually stain readily with this dye *intra vitam*. Accoyer (1924) had already suggested, on insufficient evidence, that there are two characteristic inclusions in the cytoplasm of animal cells: mitochondria, and vacuoles staining with neutral red.

It has been the policy of my associates and myself to follow the lead of Parat by basing our evidence as to structure mainly on observations on the living cell. We differ from Parat in laying stress on the lipoid material inside which the vacuole appears, and which afterwards forms an externum around it.

The Golgi element is no longer inaccessible to investigation by strictly scientific methods, and it is legitimate to suggest that the reaction against its study has now gone too far. Many cytologists are delving into the submicroscopic morphology of the cell, while a relatively enormous object stares them in the face, unnoticed. There is no intention here to try to detract from the value of submicroscopic investigation, but too many workers are prejudiced against devoting themselves to the Golgi element by reasons that are no longer valid.

A great deal of the mystery surrounding the Golgi element disappears if we look at it in the following way. When a secretion droplet is formed inside a cell, it does not usually appear loose in the cytoplasm. On the contrary, a vacuole first appears within a lipoid particle, and this vacuole grows until the lipoid is seen as a sheath round it; the secretion-product accumulates within the vacuole, and the sheath is at last thrown off.

Three main problems await solution:

1. What is the chemical constitution of the substance that forms the simple Golgi body and the externum round the vacuole? (See p. 304.)
2. What is synthesized within the vacuole in cells that make no obvious secretory product?
3. Does the simple Golgi body ever arise *de novo* in the cytoplasm? If not, do simple Golgi bodies always arise from pre-existing simple Golgi bodies? or do they arise from fragments of externa dropped off from fully formed secretion-product?

With regard to the last question, I have refrained from making Text-fig. 1 hypothetical by bringing an arrow round from the sheath fragments adhering to the secretion-product to the simple Golgi bodies, and thus completing a cycle. I have made some observations that bear upon this problem. In an unfed mouse, the amount of sudanophil material in the Golgi element of the pancreas is very small. Inject the animal subcutaneously with pilocarpine, and an hour and a half later there is a great quantity of sudanophil material in the form of Golgi bodies, many of which are large and multivacuolate and resemble the figure given by Worley (1944) of a Golgi body in a living pancreas-cell of the frog tadpole, stained by methylene blue. It is clear that there has been a great synthesis of lipoid, preparatory to the synthesis of zymogen. A few hours later the cell is stuffed with zymogen droplets, with only a fragment of sudanophil material still attached to their surfaces here and there. It is clear that a rapid lipoid-metabolism is in progress. It is very difficult to design a method of following the details of that metabolism, but whoever succeeds in doing it in any cell will have solved the main problem of the Golgi element.

ACKNOWLEDGEMENTS

My colleagues, to whom I owe a lot, are mentioned in the first paragraph and elsewhere in this paper.

Dr. Su-Hsuen Wu came to Oxford to make a thorough reinvestigation of the Golgi element in the primary spermatocyte of the snail, in collaboration with myself. Almost the whole of our joint work was done on living cells. It was intended that we should both continue the work by studying fixed material after her return to China; but she must have reached Pekin just before the city was captured, and I have not been able to get in touch with her. I earnestly hope that I may be able to collaborate with her again one day. Meanwhile I can only pay tribute to the remarkable perseverance and independence she showed in her determination to arrive at the truth about the nature of the object investigated. Under the circumstances I believe that she would wish me to publish our results. Whatever there may be of value in the account of the living primary spermatocyte of the snail is due more to her than to me.

We have received all possible help and encouragement from Professor A. C. Hardy, F.R.S. I must particularly mention Dr. G. C. Hirsch. His book (1939) has long been a source of inspiration; collaboration and discussion with him have been of immense benefit. I thank also Miss O. Wilkinson and Miss M. Le Bechée for much skilful practical assistance, willingly given.

SUMMARY

1. The Golgi element has been reinvestigated in the same kinds of cells as were the subject of the author's 1944 paper.

2. Two new methods have been used, namely, phase-contrast microscopy and an improved form of the sudan black technique, in which the tissues are postchromed at 60° C.

3. The Golgi element consists of separate bodies, spheroid in shape. These Golgi bodies may be simple (i.e. non-vacuolate), or may contain one or more vacuoles. The material of the simple Golgi body and of the externum of the vacuolate body is a lipoid that in some cases can be shown to contain lipine. The secretion-product of the Golgi body originates in the vacuole.

4. The opinion as to the structural plan of the Golgi element set out in the earlier paper has been confirmed in the main. There are, however, two exceptions to this:

(*a*) The vacuole in the Golgi body does not invariably colour with neutral red, and this dye occasionally causes the appearance of vacuoles not present before, both within the Golgi region and in other parts of the cytoplasm.

(*b*) 'Diffuse lipoid' is not a characteristic feature of the Golgi element.

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Aldehydes in relation to Absorption of Fat from the Intestine and Metabolism of Fat in the Liver

BY

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With one Plate

INTRODUCTION

THAT aldehydes may be concerned in the synthesis and degradation of fat has often been speculated upon in the past. For example, the intervention of the aldol condensation

$$R.CHO + CH_3.CHO \rightleftharpoons R.CH(OH).CH_2.CHO \qquad (1)$$

is compatible with most of the known facts about fat metabolism. In particular, it offers a ready explanation of the fact that the natural fatty acids have, almost invariably, an even number of carbon atoms, and of the fact that degradation of these acids normally occurs by loss of two carbon atoms at a time. But attempts to discover acetaldehyde in tissues, in relation to fat metabolism, have not been successful, and there are very few observations in the literature on the occurrence of longer-chain aldehydes. However, recent studies have shown that acetyl phosphate, $CH_3.CO.OPO_3H_2$, is a common constituent of tissues. The absence of resonance in this molecule (compared with that possible in free acetic acid) suggests the possibility that acetyl phosphate, or a related radicle, could replace acetaldehyde in equation (1). This being so, it becomes of great importance to investigate the occurrence of long-chain aldehydes in tissues, in relation to fat metabolism. In this paper attention is focused on the liver of the rat and the mouse. In this organ there is a close cytological association between sites of fat metabolism and sites of long-chain aldehydes. Failure to appreciate this in the past was probably caused by the ease with which oxidation of aldehyde to the corresponding fatty acid occurs during the usual procedures of the biochemist. By the use of cytochemical methods this difficulty is largely obviated.

In addition, preliminary experiments suggested that there is a relationship between aldehyde formation and the absorption of fat by the intestinal mucosa. Studies were therefore made of this point.

CYTOCHEMICAL METHODS

Tissues were studied for the distribution of alkaline phosphatase by the method of Takamatsu (1939) and Gomori (1939), as revised by Danielli (1946), and for the distribution of long-chain aldehydes by the method of Feulgen and Voit (1924) as revised by Danielli (1949). For demonstration of

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phosphatase paraffin sections were cut at 5 μ, and for demonstration of aldehyde frozen sections were cut at 10 μ and 20 μ with liver, and at 15 μ with intestine.

Fat was stained in the frozen sections with Scarlet R (Gurr), using the stain in 70 per cent. alcohol for 1 hour at room temperature, and differentiating in 70 per cent. alcohol. This method can be used successfully after carrying out the cytochemical test for aldehydes. Counterstaining with toluidine blue was found useful.

TREATMENT OF ANIMALS

The animals used were rats and mice. When placed on a special diet they were weighed daily. The normal diet contained carbohydrate, fat, and protein. The special carbohydrate diet contained 76 per cent. sucrose, 15 per cent. agar, 5 per cent. yeast, 4 per cent. salt mixture: animals on this diet were given a supplement of vitamins A, D, and E daily. All animals, including those starved, were allowed free access to water.

THE ABSORPTION OF FAT FROM THE INTESTINE

When animals were kept on a normal diet, which contained rather a small amount of fat, only occasional fat droplets were found in the intestinal mucosa. The intracellular droplets were covered with a spherical shell of aldehyde, as also were those droplets observed in the lacteals. In addition there was frequently a diffuse distribution of aldehyde between the nucleus and basement membrane of the cells making up the intestinal epithelium. When animals were kept on the carbohydrate diet, the intestinal mucosa was comparatively lacking in aldehyde and, as would be expected, no fat was found in the epithelial cells or in the lacteals. These preliminary observations suggest that aldehydes might be involved in the absorption of fat by the intestinal mucosa.

To test this hypothesis, animals which had been starved overnight were given by stomach tube a dose of 1 ml. of an emulsion of olive oil in water. The emulsion contained 50 per cent. of olive oil, with sufficient sodium bicarbonate to give a relatively stable emulsion. These animals were killed at intervals of 1, 2, 4, and 6 hours after administration of fat. When frozen sections from the intestines of these animals were examined, the intestinal mucosa cells showed a very heavy absorption of fat, especially in the case of those animals which were killed after 4 hours and 6 hours. It was commonly found that different regions of the epithelium would show quite different stages in the absorption of fat. In some cases the cells and lacteals were free from fat except for a few droplets just inside the free border of the cells; in some cells droplets were to be observed throughout the cell, in others the greatest concentration of droplets was proximal to the basement membrane; in some regions of the intestine fat droplets would be observed in the mesenteries only. When these sections were stained, aldehyde was found only in animals which were killed a short time after administration of fat. It thus was clear that the absorption of triolein does not occur by any stage involving the intervention of aldehydes. The fact that aldehyde was to be observed only in the animals which were killed soon after dosing suggests that the aldehyde is formed in the mucosa cells in connexion with processes other than the absorption of fat, that when fat enters the cell the long-chain aldehydes are adsorbed upon the surface of the fat droplets, and are carried out of the cell by the fat droplets as these droplets pass into the lacteals; i.e. the absence of aldehyde in the intestinal mucosa cells towards the end of the experiment is probably attributable to a process of washing-out of aldehyde by the passage of oil droplets through the cell.

Whilst it seemed clear that aldehyde is not involved in the absorption of triglyceride, there still remained the possibility that it might be concerned with the absorption of free fatty acid. To test this animals were given 1 ml. of a suspension of sodium oleate in water; the suspension contained 50 per cent. of oleic acid by volume, adjusted to pH 6 by addition of sodium hydroxide. These animals were killed 1, 2, 3, 3½, 4, 4½, 5, and 5½ hours after administration of the fatty acid. The results obtained did not differ substantially from those found with triolein.

The conclusion thus appeared that long-chain aldehydes are not involved either in the absorption of fat from the intestinal lumen, nor in the passage of fat through the intestinal cells.

The Metabolism of Fat in the Liver

When the livers of animals on a normal diet were examined for the occurrence of aldehyde and of fat, it was found that the aldehyde was distributed around the intra-cellular fat droplets as a spherical shell. In addition there would often be a diffuse distribution of aldehyde in the cytoplasm, and sometimes this diffuse aldehyde was present in cells which appeared to contain no fat droplets. Considerable areas of the liver are often quite free from aldehyde. It was not clear, from these observations on animals on normal diet, whether the occurrence of the aldehyde was to be associated with the synthesis of fat or with the degradation of fat. Experiments were therefore carried out with animals fed on a diet which was rich in fat, on animals fed on a diet containing no fat but high carbohydrate, and on animals which were starved.

When the diet contained a considerable amount of fat, all the droplets found in the liver were surrounded with a spherical shell of aldehyde. This was also true of animals which had been starved for periods of up to 6 days. In the case of the starved animals it is known that the fat droplets appearing in the liver are derived from the fat depots, from which they pass to the liver. In the liver the fat is broken down into carbohydrate. Thus from these experiments it was clear that aldehyde is closely associated with the breakdown of fat to carbohydrate.

When animals are kept on a carbohydrate diet of the type used here, containing no fat, it is known that in so far as fat appears in the liver it is formed by synthesis of fat from carbohydrate. Experiments with tagged fat have shown that no fat passes from the fat depots to the liver, but fat may be synthesized in considerable amounts in the liver from carbohydrate. In the experiments reported here, the usual response to placing an animal on carbohydrate-rich diet was a great decline in the quantity of aldehyde found in the liver. This was commonly associated with a parallel decline in the amount of fat to be observed in droplet form in the liver cells. After 2 or 3 days on this diet, aldehyde may begin to appear again in the liver. The greater part of this aldehyde is distributed diffusely in the cells, and not always in those hepatic cells containing fat droplets. The reappearance of aldehyde, however, was closely associated with the reappearance of droplet fat in the liver. The fat droplets frequently had spherical shells of aldehyde, but on the other hand it was quite common to find droplets which had no such shell of aldehyde. In some animals there was evidence of the secretion of aldehyde into the bile canaliculae.

It seems clear from these experiments that aldehyde is intimately concerned in the breakdown of fat, and may also be concerned in the synthesis of fat from carbohydrate, although the evidence for the latter conclusion is not so satisfactory as for the former.

Studies were made of the cytochemical distribution of alkaline phosphatase during fat synthesis and degradation in the liver. No evidence was obtained which would suggest that this enzyme is concerned in fat metabolism.

In some instances it was found that the bile canaliculae contained a concentration of aldehyde. This aldehyde is presumably secreted into the bile canals by the hepatic cells. The function of this activity is at present obscure.

In all experiments the fatty nature of the aldehyde was tested by extraction of the tissue sections with acetone before the performance of the cytochemical reaction. In all cases the aldehyde was extracted by acetone, showing that it is a fatty compound.

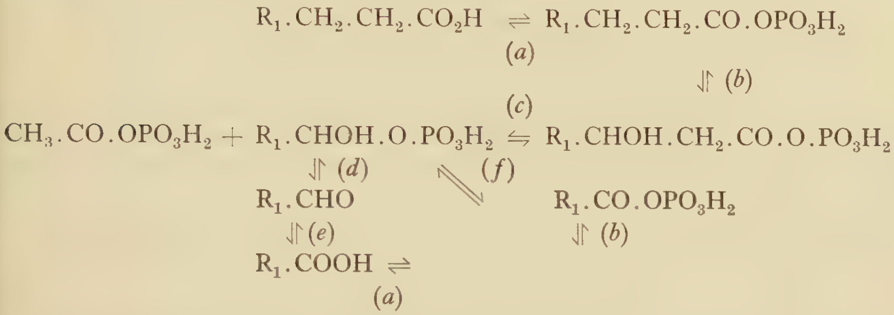
Discussion

The results obtained in this work show that whilst long-chain aldehydes are probably not concerned in the absorption of fat from the intestine, they are intimately concerned with fat metabolism in the liver. Studies of a variety of tissues grown in tissue culture have shown that when fat is being laid down in cells, it is always surrounded by a spherical shell of aldehyde. These results will be published separately. The main conclusion to be drawn is that aldehydes are likely to be an essential intermediate in fat metabolism.

At present the details of the processes involving the synthesis and degradation of the higher fatty acids are obscure. It is known from extensive classical studies that a process of β oxidation is commonly involved, so that a series of 2-carbon fragments is lost from the fatty acid chain, rather than carbon dioxide. It is also known from recent work, particularly that of Lehninger (1945), that whereas free fatty acids are not readily attacked by the enzyme systems which can be extracted from hepatic cells, the acylphosphates are readily oxidized and are probably a main intermediate. To these conclusions must now be added the probability that the long-chain aldehydes are also a main intermediate.

As was pointed out in the introduction, it may be that not free aldehydes but phosphorylated derivatives of aldehydes are the true intermediates. If this is so the aldehyde found in the experiments reported here may be a breakdown product of an aldehyde phosphate. Aldehyde phosphates such as $R.CHOH.OPO_3H_2$ would certainly be unstable. Therefore two possibilities must be considered: (i) that free long-chain aldehydes are true intermediates; (ii) that free long-chain aldehydes are by-products or end-products of a synthetic cycle.

In case (i), in the degradation of a long-chain fatty acid it is possible that aldehyde phosphates and free aldehydes are involved in a series of reactions such as (*a*) → (*b*) → (*c*) → (*d*) → (*e*) below

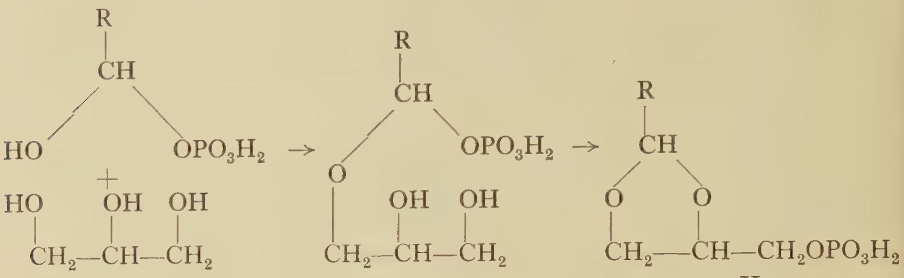


$$\begin{tikzcd}
 & R_1.CH_2.CH_2.CO_2H \arrow[r, leftrightarrow, "(a)"'] & R_1.CH_2.CH_2.CO.OPO_3H_2 \arrow[d, leftrightarrow, "(b)"] \\
CH_3.CO.OPO_3H_2 + & R_1.CHOH.O.PO_3H_2 \arrow[r, leftrightarrow, "(c)"] \arrow[d, leftrightarrow, "(d)"] \arrow[dr, leftrightarrow, "(f)"] & R_1.CHOH.CH_2.CO.O.PO_3H_2 \\
 & R_1.CHO \arrow[d, leftrightarrow, "(e)"] & R_1.CO.OPO_3H_2 \arrow[d, leftrightarrow, "(b)"] \\
 & R_1.COOH \arrow[r, leftrightarrow, "(a)"'] & {}
\end{tikzcd}$$

Alternatively, in case (ii) instead of the occurrence of reaction (*d*), which involves liberation of free aldehyde, aldehyde phosphate may be directly oxidized to fatty acid phosphate as indicated by (*f*). In the latter case free aldehyde would probably only appear as the termination of a series of synthetic reactions. A technique for the cytochemical demonstration of aldehyde oxidase would yield results which would assist in considering the likely sequence of intracellular reactions. At present no such technique is available. But it is known that there is a very high concentration of aldehyde oxidase adsorbed on the surface of freshly secreted milk fat droplets (Worden, 1943). It seems very unlikely that this is a fortuitous association. Furthermore, Prof. A. Worden informs me that there is also phosphatase adsorbed on the surface of milk fat droplets, which suggests a dephosphorylation is involved in at least one stage in fat synthesis. On the other hand, cytochemical studies made in the course of this work have failed to show any significant correlation between the occurrence of alkaline phosphatase and of fat metabolism.

A point which may prove to be significant was that each fat droplet undergoing degradation in the liver is surrounded by a spherical shell of basiphilic material, the site of which roughly coincides with the shell of aldehyde. The basiphilic character may be caused by nucleic acids, phospholipines, &c.

In conclusion it should be pointed out that, when it is realized that long-chain aldehydes or their derivatives are essential intermediates in fat metabolism, a new significance attaches to the occurrence of the aldehyde acetals which were discovered by Feulgen and Bersin (1939) and which were discussed in a previous paper (Danielli, 1949). These acetals will arise, for example, by condensation of glycerol with aldehyde phosphate:


I II

The condensation leading to substance I may well go spontaneously, since aldehyde phosphates would be highly reactive, and the wandering of the phosphate group to give II (the substance isolated by Feulgen *et al.*) may also be spontaneous, since it closely resembles the wandering of the phosphate group in the reaction α glycerophosphate $\rightleftharpoons$ β glycerophosphate. It is reasonable to suppose that these acetals are intermediates in the synthesis of triglycerides and the phospholipins, &c., although the possibility must be borne in mind that the acetals may be also essential intermediates in the main channel of fatty acid metabolism.

I am indebted to Dr. I. J. Lorch for assistance with the experiments, and to Prof. G. A. R. Kon and Prof. A. Haddow for their comments on the manuscript. The investigation has been assisted by grants made to the Royal Cancer Hospital by the British Empire Cancer Campaign, the Anna Fuller Fund, and the Jane Coffin Child Memorial Fund for Medical Research.

SUMMARY

1. During the absorption of fatty acids or of fat from the intestine of mice and rats, no intervention of long-chain aldehydes occurs.

2. When fat is being metabolized or synthesized in the liver of mice and rats aldehydes are prominent. Each fat droplet being metabolized is surrounded by a spherical shell containing aldehyde and basiphilic material.

3. The results indicate that long-chain aldehydes or their derivatives are essential intermediates in the metabolism of fatty acids, and suggest that the acetals of these aldehydes are intermediates in the synthesis of glycerides and phospholipines, &c.

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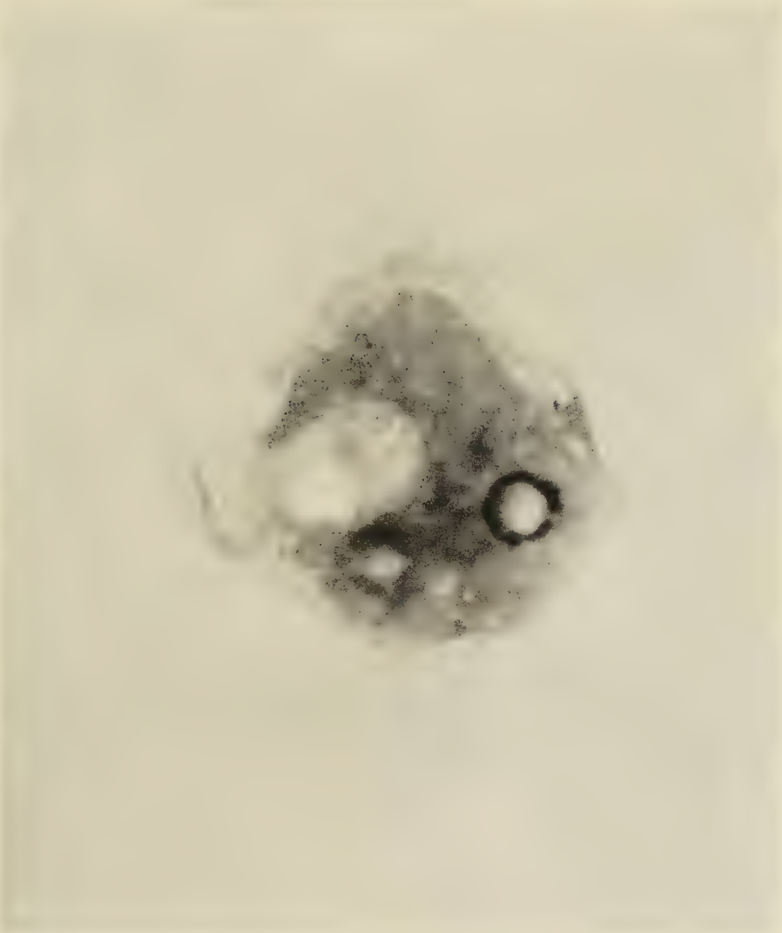


Shows aldehyde in a single hepatic cell in a frozen section. The surrounding cells are aldehyde-free. The large clear central body is the nucleus. The shell of aldehyde appears as a ring surrounding a large fat droplet which is in focus. Another large fat droplet is out of focus, and there are many small droplets and some diffuse aldehyde

J. F. DANIELLI—PLATE I

The Histochemical Detection of Beryllium

BY

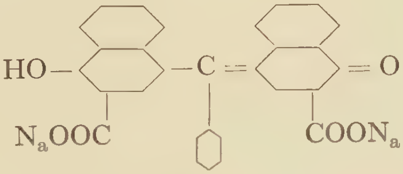
F. A. DENZ

(From the Experimental Station, Porton, Wilts.)

DURING an investigation of the toxic action of beryllium salts on experimental animals a histochemical test was developed to provide information on the distribution and site of action of beryllium. The technique is reported as an example of the application of chemical methods to histological problems.

The reactions of beryllium salts with dyes of the triarylmethane series have been studied by Aldridge and Liddell (unpublished) in the course of developing a more satisfactory method for the chemical determination of beryllium in biological material. From this work it appeared probable that the coloured lakes formed by beryllium and dyes of the naphthochrome series would be suitable for the detection of the element in histological sections. A small number of naphthochrome dyes were tested for laking properties with concentrations of beryllium varying from 1 to 10 μg. per ml. in phosphate buffers ranging from pH 4·5 to 9·0. The dyestuff naphthochrome green B at pH 5 forms with beryllium an apple-green lake that contrasts with the blue-black colour of the dyestuff. Iron and aluminium share with beryllium the property of forming lakes with naphthochrome green B, but these lakes differ in colour. The ferric lake is dark blue-green and the aluminium lake is yellowish-green in colour. Further, the optimum pH for lake formation differs for the three elements. The colour reaction between beryllium and naphthochrome green B is maximal at pH 5 but iron and aluminium at this pH produce very slight colour changes, and these only at relatively high concentrations. In this way by selecting the dyestuff and pH range, it is possible to find conditions which permit a characteristic colour change with beryllium, but inhibit any reaction with other elements such as iron and aluminium.

Napththochrome green B (C.A.C.) has Schultz Number 851 and is the sodium salt of pheno-oxy-dinaphtho-fuchsondicarboxylic acid. Its structural formula is



Compounds of this type react with polyvalent metallic ions to form chelate compounds linking the metal to the adjacent hydroxy and carboxylic groups.

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It is probable that the coloured lake formed between naphthochrome green B and beryllium is produced by chelation.

METHOD

Two solutions are used.

1. 0·5 per cent. aqueous solution of naphthochrome green B. This must be freshly made immediately before use.
2. Phosphate buffer adjusted to pH 5.

PROCEDURE

Tissue is fixed in formol alcohol or formol saline, and passed through alcohols to xylene (or cedar wood oil) before paraffin embedding. The paraffin sections are treated with xylene and brought to water through alcohols.

Equal volumes of the naphthochrome solution and the phosphate buffer are mixed immediately before use. This solution does not keep more than a few hours. Stain slides for 30 minutes in a Coplin jar, the solution being maintained at 37° C. in an incubator during staining. At temperatures below 37° C. the intensity of staining decreases considerably. Wash in distilled water and differentiate by immersion directly in absolute alcohol for 30 minutes.

Wash in distilled water.

Counterstain with 1 per cent. aqueous solution of acridine red for 5 minutes.

Wash in distilled water, differentiate rapidly in absolute alcohol, pass directly to xylene and mount in Canada balsam.

The sites of beryllium deposits stain a clear apple-green against the red background of the counterstain.

BIOLOGICAL STANDARDIZATION OF THE TEST

Intradermal Injections and Skin Titrations

The method of intradermal injection was used to provide material containing known amounts of beryllium. Doses from 1 μg. to 1 mg. of beryllium as 0·1 ml. of a solution of beryllium lactate were injected intradermally into the shaven backs of white rabbits. The lactate was chosen for this work, because the aqueous solution of this salt is near neutrality (pH 5·5) and the lactate ion is unlikely to cause any physiological disturbance. At intervals from 4 to 24 hours after inoculation the animals were killed and the injected areas were excised. The beryllium lake in the skin could be seen clearly in stained sections with inocula of 50 μg. and the lowest detectable dose was 3 μg. In addition to indicating the range of sensitivity, the skin titrations provided a rough set of colorimetric standards for comparison with tissues from animals injected intravenously with beryllium salts.

The same method was used to test the stain against the salts of iron and aluminium. In all comparisons of the staining properties of these salts and the salts of beryllium equimolar solutions were used. Solutions of ferric alum and aluminium sulphate were injected intradermally in white rabbits.

Subsequent staining showed that these elements could be detected only in concentrations at least ten times greater than beryllium. Furthermore, the almost complete absence of staining of tissues obtained 24 hours after inoculation showed that both iron and aluminium ions are rapidly removed from the site of injection. By contrast, beryllium remains at the site of inoculation in a stainable form for at least 3 months. It was concluded that iron and aluminium would not interfere because neither could be present in a concentration great enough to form a visible lake with the dyestuff. This conclusion has been confirmed by further animal experiment. Lake formation did not occur in the tissues of any control animals except in the pancreas, which stains a diffuse pale green colour.

Intravenous Injection in Animals

The size of the dose for intravenous injection and histological tracing was found by injecting the warmed tail vein of mice with doses of beryllium containing from 5 to 1,000μg. of beryllium. The LD 50 for mice is 20μg. of beryllium and it is naturally desirable that the tracing dose should be of this order. In animals killed 2 hours after injection strong staining was found following injections of 200μg. of beryllium, but below this level the distribution of beryllium in the tissues was difficult to see. The level of 200μg. of beryllium was chosen as optimal and the histological changes were followed up to 8 hours after injection, the mice being killed at regular intervals. Following injections of 200μg. mice die within 24 hours, but after 50μg. survive for 2 or 3 days. These later stages were followed in a further experiment at the reduced dosage. Fiftyμg. was the least amount of beryllium that could be satisfactorily followed in the tissues of the mouse, even after considerable experience had been gained from the study of material from mice injected with 200μg.

Utilization of the Method

The histochemical findings will be reported in detail elsewhere as part of an account of the toxicity of beryllium. An outline of the findings is given to illustrate the potentialities of such a method. By the naphthochrome method beryllium can be seen within the larger blood-vessels of all organs of the body for 15 minutes following intravenous injection of a soluble salt of beryllium. It then disappears from the general circulation and concentrates in the sinusoids of liver and spleen and in the vasa recta of the kidneys.

The development of the pathological lesions can be associated with the focal concentration of beryllium. In the liver where the overall concentration may be of the order of 5μg. per gramme of tissue the focal concentration in the sinusoids is equivalent to 100–200μg. per gramme. Around such concentrations the liver cells are destroyed and the characteristic focal necrosis of beryllium poisoning develops.

Beryllium in tissues and in blood appears to be attached to protein. This union accounts for its persistence at the sites of injection, and the sharpness with which it stains. The formation of focal concentrations within sinusoids is a most important factor in enabling the tracing to be carried out. Fifty μg. of beryllium uniformly dispersed throughout the tissues of a mouse would produce a concentration of about 2 μg. per gramme and this is below the limits of histochemical detection by the naphthochrome method. However, following the injection of 50 μg. of beryllium focal concentrations within liver, spleen, and kidney reach values fifty times as great as this and are readily seen.

Pathological change follows focal accumulation of beryllium and a combination of the naphthochrome method and routine histological methods has enabled such problems as the development of midzonal liver necrosis and circulatory changes in the kidney to be studied.

LIMITATIONS AND DIFFICULTIES

Effect of Solubility of Beryllium Compounds

The test is specific for beryllium but the dyestuff does not react with all compounds of beryllium. The refractory oxide and the silicate are insoluble in water, resist the action of acids, and do not react with naphthochrome green B. *In vitro* lake formation with naphthochrome green B occurs only with the beryllium ion but the situation in tissues is more complex. If dilute solutions of beryllium salts are mixed with serum the beryllium cannot be separated from the serum proteins by ultrafiltration and in general beryllium ions appear to combine rapidly with the tissue proteins. This union is firm enough to retain beryllium at the site of subcutaneous injection for months but does not prevent the reaction between beryllium and naphthochrome green B.

If the refractory oxide or the silicate is injected into the skin, solution of the injected material takes place very slowly so that after some weeks faint green staining of the inoculation site is obtained with naphthochrome green B. This occurs only when the inoculum is large and the tissue reactions have been going on for a long time. When the insoluble compounds are widely dispersed in the tissues after inhalation of dusts or following the intravenous injection of suspensions the histochemical test does not disclose the localization of the oxide or silicate particles. The test is of value only for the study of the reaction of tissues to the injection of soluble compounds.

Dosage Level

The optimum tracing dose was found to be several times the LD 50 and one of the difficulties, that of early death of the animal, has already been mentioned. A further difficulty is evident when the possibility that the distribution of the higher doses in tissues may be different from that of lower doses is considered. This is certainly the case in the mouse lung. At an inoculation level of 200 μg. of beryllium the lungs stain strongly and the staining persists for 8 hours at least, whereas with 50 μg. staining is never diffuse, is always slight and entirely disappears within an hour of injection. Again beryllium was present in the medulla of three out of four adrenals from animals injected at the higher and only in one of twenty-three at the lower level. In this investigation the assumption was made that where the distribution of beryllium within the organs was constant in a large series of animals at both levels of injection, i.e. at 200 and at 50μg., this distribution was likely to be present at even lower levels. This assumption was tested against pathological findings and the results of chemical analysis and was not found to be incorrect.

Contamination

Owing to the sensitivity of the staining method contamination is a factor that must be considered in the handling of histological material. Contamination by iron, aluminium, or beryllium salts must be avoided. Fine specks of rust give an intense colour reaction. Care must be exercised during dissection of the injected animal. If the beryllium-rich liver is cut before tissues such as thymus or muscle which do not contain beryllium, sufficient may be carried over on the knife to give a considerable degree of diffuse staining in the latter organs. A fresh knife should be used for each organ. The tissues may, however, be fixed in a common pot.

Artifacts

Those due to contamination have been mentioned above. The staining is often more intense at the margin of the section than at the centre. Occasionally the margins of control and experimental tissues are stained a diffuse green. This is usually but not always due to overlong staining, and in some cases the cause has not been found. The artifact is sufficiently obvious to prevent confusion with the staining of the beryllium within the tissues. The exocrine tissue of the pancreas usually takes on a blue-green tint that is not extracted by alcohol. This colour is slight and diffuse and readily distinguished from the focal apple-green staining of beryllium deposits.

ACKNOWLEDGEMENTS

This work owes much to the help and encouragement of Dr. J. M. Barnes and Mr. W. N. Aldridge who have been so closely associated with it that I find it impossible to separate their contributions and mine. I wish to acknowledge the assistance of Mr. H. F. Liddell whose exceptional knowledge of dyestuffs made this work possible.

SUMMARY

A histochemical test for beryllium in paraffin sections is described. A green lake that is formed with beryllium by the dyestuff naphthochrome green B is specific for beryllium under the stated conditions. The test works only with the soluble compounds of beryllium. Deposits of as little as 3μg. of beryllium can be detected and the distribution in the tissues of mice can be followed after the intravenous injection of 200μg. of beryllium. The applications and limitations of the method are discussed.

A Simple Method for Phase-contrast Microscopy: Improvements in Technique

BY

JOHN R. BAKER, D. A. KEMPSON, AND P. C. J. BRUNET

(From the Department of Zoology and Bureau of Animal Population, Oxford)

IF the condenser of a microscope is first set in its so-called 'critical' position and then gradually lowered, the real image of the source of light will be brought down from the plane of the diaphragm of the eyepiece till it reaches a position just behind (above) the back focal plane of the objective. If a piece of black paper, from which a ring-shaped strip has been cut away, is held immediately in front of the source of light, a bright image of the annulus can easily be thrown just behind the back focal plane of the objective by lowering the condenser to the appropriate degree. If now a phase-plate, provided with an annulus, is placed in the same plane as the image of the illuminating annulus, and if the phase-plate annulus is of such dimensions that it coincides with this image, phase-contrast microscopy will have been achieved.

This method of phase-contrast microscopy was suggested by one of us in a paper on oblique illumination (Baker, 1948). It was worked out practically by Kempson, Thomas, and Baker (1948). Full instructions were given on the adaptation of an ordinary microscope for phase-contrast work. The method requires no special objectives and no special apparatus for centring the illuminating annulus. It is therefore extremely simple and inexpensive.

The purpose of the present paper is to record certain improvements in the method. Still further simplification has been achieved, while the results obtained are better than before.

In the earlier paper we gave exact instructions for using phase-contrast with a Zeiss apochromatic water-immersion objective. We now think it best to give instructions for using phase-contrast with lenses that are readily available in this country. We have confined ourselves to the products of Messrs. W. Watson, as follows:

Objectives

4 mm. (1/6th inch) 'Parachromatic', N.A. 0·7, dry,
4 mm. (1/6th inch) 'Parachromatic', N.A. 0·8, dry,
1·8 mm. (so-called 1/12th inch) 'Versalic', oil-immersion.

Condensers

Ordinary two-lens Abbe,
'Parachromatic'.

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Full details of the proper sizes for all the parts of the apparatus, when these lenses are used, are given in Table 1. It was thought convenient to collect all the numerical data in a single table, so that they need not be given elsewhere.

The reader is referred to the earlier paper for a general account of the method adopted. In the present paper we restrict ourselves to the improvements we have made since that paper was written.

Illuminant. We have found the best illuminant to be a powerful electric bulb with a 'porcelain-processed' surface. 'Flashed-white' bulbs are quite good; 'opal' ones have the advantage of a very homogeneous surface, but unfortunately this absorbs much of the light, so that they are not suitable for high-power work. 'Pearl' bulbs are inferior, and coarsely frosted ones are to be avoided. The best bulb known to us is the 150-watt 'Helios' porcelain-processed enlarging bulb, intended for use in photographic enlargers. These are made with the whole surface or the tip alone processed. We use the bulb made for 200–220 volts, but overrun it on the mains at 230 volts. We no longer use a bull's-eye condenser.

Illuminating annulus. The simplest way of making this is to cut an annular strip out of a sheet of black paper, and to glue both the central and the outer part of the sheet on to a piece of plain glass in such a way as to leave an annular space of uniform width between them. However, the heat from the electric bulb may crack the glass and we therefore prefer to use brass. The central disk is fixed to the outer piece by four narrow radial spokes, which traverse an annular empty space and are soldered to the brass at each end. The illuminating annulus should be placed close to the electric bulb.

The appropriate sizes for annuli (outer and inner radii) are shown in Table 1. The size necessary to produce coincidence between the phase-plate annulus and the image of the illuminating annulus is dependent on five factors: (1) the distance of the illuminating annulus from the condenser (via the mirror); (2) the focal length of the condenser; (3) the focal length of the objective; (4) the distance of the phase-plate from the back focal plane of the objective; and (5) the radii of the phase-plate annulus. The image of the illuminating annulus should theoretically coincide exactly with the phase-plate annulus. However, any straying of the image of the illuminating annulus beyond the limits of the phase-plate annulus is very damaging to contrast, while the reverse causes little deterioration in the image. For this reason we generally make our illuminating annulus of such dimensions that the image of it is slightly narrower than the phase-plate annulus, the edges of which can be seen outside and inside the image of the illuminating annulus.

The first image of the illuminating annulus (below the object) is a long way from the aplanatic plane of the objective, and the second image (in the plane of the phase-plate) is therefore imperfect: that is, the outer and inner edges of the image are not in exact focus at the same time. We do not find that this causes any observable detriment to the image of the object as seen through the eyepiece of the microscope.

TABLE I

Objective				*Condenser*			*Phase-plate*				*Illuminating Annulus*			
									Radii of annulus			*Radii*		
Name	*Focal length*	*N.A.*	*Radius of back lens*	*Name*	*Focal length*	*N.A.*	*Distance above back lens of objective*	*Total radius*	*Outer*	*Inner*	*Distance from bottom lens of condenser*†	*Outer*	*Inner*	*Column Number*
Parachromatic	4	0·7	2·6	Abbe	12	'1·2'*	1·1	3·0	1·0	0·7	160	21	16	1
,,	,,	0·8	2·9	,,	,,	,,	4·1	3·5	1·1	0·75	180	26	18·5	2
,,	,,	,,	,,	Parachromatic	7	0·9	,,	,,	,,	,,	190	,,	,,	3
Versalic	1·8	1·28	2·25	,,	,,	,,	2·2	2·75	0·9	0·63	210	19	15	4
,,	,,	,,	,,	,,	,,	,,	0·3	2·38	,,	0·7	190	,,	,,	5
,,	,,	,,	,,	Abbe	12	'1·2'*	,,	,,	,,	,,	280	21	16	6
,,	,,	,,	,,	,,	,,	,,	2·2	2·75	,,	0·63	360	19	15	7

All linear measurements are given in millimetres.

The objectives and condensers listed in this Table are made by Messrs W. Watson.

The annuli of the phase-plates are generally smoked so as to allow the transmission of 75 per cent. of the light falling upon them.

* Not aplanatic.

† Via the centre of the mirror.

Mirror. A stainless steel mirror, or any other kind that gives only a single reflection, is slightly preferable, but an ordinary microscope-mirror gives satisfactory results.

Colour-filter. A filter, giving maximum transmission at 500mμ, should be used when photographs are taken. For visual work we generally prefer to use no filter, since to the human eye the yellowish light of the electric bulb gives a better contrast against grey and black than does green, and this appears to us generally to outweigh the optical advantages of illumination with a wave-band closely adjusted to the thickness of the magnesium fluoride on the phase-plate.

Condenser. The chromatically and spherically corrected Parachromatic condenser presents two advantages over the Abbe: objects are seen without any tendency to adventitious colour, and the phase-effect is obtained right up to the edge of the field of view. When the Abbe is used, the phase-contrast is not good near the edge of the field of view. Nevertheless, quite good results are obtained with the Abbe, and no one should be dissuaded from using the method simply because he has not got a corrected condenser.

Phase-plate holder. We no longer use spring jaws, but simply cement the plate in the holder. The inner surface of the holder must be blackened.

Phase-plate. The radius of the phase-plate must be at least as great as that of the black lens of the objective.

If the back focal plane of the objective lies within its lens-system, the phase-plate should be placed close behind the back lens. If the back focal plane of the objective lies behind (above) the back lens, the phase-plate should be placed a millimetre or two behind (above) this plane.

The thickness of the magnesium fluoride film deposited on the lower surface of the phase-plate should be such that it retards light to the extent of approximately a quarter of a wave-length of apple-green light, in comparison with light travelling through a film of air of equal thickness. (Apple-green light is chosen because it lies near the middle of the spectrum of the light used for illumination.) Our phase-plates are optically flat circles about a millimetre thick. They are made by Messrs. R. & J. Beck, who also deposit the magnesium fluoride upon them. Care should be taken to check the thickness of the deposit. This can be done by holding the plate over a dark background and looking at it in such a way that the line of vision subtends an angle of approximately 30° to a line perpendicular to the surface of the plate. If the retardation is exactly $\frac{500}{4}$mμ, the interference colour seen in daylight at this angle will be blue-green (in the second order of Newton's rings). Our experience is that the very best results are obtained when the film is slightly thinner than this, so that the colour given is blue.

In our earlier paper we described the making of a 'negative' or bright-contrast plate, that is, a plate in which the annulus consists of a *wall* of magnesium fluoride standing up from the glass. We now find that for nearly all objects 'positive' (dark) phase-contrast is preferable. The annulus is therefore excavated in the magnesium fluoride to the level of the glass, and exists as a *ditch* instead of a wall. To measure and mark off the annulus to be cut in the coating, a micrometer eyepiece should be slipped into the binocular used for observing the cutting operation (or into a monocular with a suitable low-power objective). The diameter of the optical flat being known, the eyepiece-scale can be calibrated accordingly and the boundaries of the annulus marked with a finely pointed needle. Owing to the small amount of scraping required to make a 'ditch' for a positive phase-plate, the method previously given for operating the tool in making a negative plate was abandoned in favour of a tool-rest. This is simply a $\frac{1}{4}$-inch rod, held horizontally and supported at each end by convenient fixtures on the bench. The rod is placed close to the phase-plate on the turntable, so that the scraper, held in the hand, rests in a V-mark filed in the rod. A rigid support is thus provided, yet freedom of movement is permitted. Care must be taken not to mark the glass with the tool by continuing to scrape longer than is necessary. Progress is easily observed if the lighting is adjusted to the correct angle and the plate frequently wiped with a grease- and grit-free handkerchief. The difference in the coated surface and the clear glass is unmistakable.

We have had our best results with phase-plate annuli of the sizes shown in Table 1. It will be observed that the annulus is placed nearer to the centre of the plate than to an imaginary circle drawn round it, equal in diameter to the back lens of the objective. This is a purely empirical finding.

The amplitude of the direct (undiffracted) light is reduced by smoking the plate over a flame and then removing the smoke from the whole surface except the annulus. For any particular object there is an ideal degree of reduction of amplitude of the direct light. In general, to suit a wide range of objects as well as possible, we find that the smoking should be of such a depth as to allow the transmission of 75 per cent. of the light reaching the annulus. The fuel used for making a smoky flame is a mixture of absolute alcohol and xylene, the latter being added until the flame gives off a slight smoke. Too much xylene causes a coarse deposit of carbon and produces irregular and streaky effects.

We nowadays generally check the depth of smoking by the use of a step-wedge. The comparison with the step-wedge is made while the whole surface of the plate is still covered with smoke. If a suitably graduated step-wedge is not available, a simpler and convenient standard can be made from Ilford 35 mm. Leica film or Kodak Super XX 35 mm. film. When the emulsion is cleaned off, the grey-tinted base has a transmission of from 60 to 70 per cent. The desired amount of smoking being 75 per cent. for the phase-plate, it is evident that the smoke density on the phase-plate should be a trifle lighter than that of the film base. The wedge or film base should be masked so that only an area of the same size as the smoked phase-plate is used for comparison.

Auxiliary microscope. The low-power lens used for centring and focusing the image of the illuminating annulus with the phase-plate should have a low numerical aperture. A cardboard stop should be inserted in the lens system if necessary.

It may be found convenient to use a special auxiliary microscope, fitting in place of the eyepiece. In this auxiliary microscope the objective is the field-lens of a low-power eyepiece, with a cardboard stop about one-third its diameter immediately above it; the eyepiece is *either* the whole *or* the eye-lens only of an ordinary high-power eyepiece. A brass tube 6 cm. long, split longitudinally, will hold the 'objective' and eyepiece together in their tubes and permit focusing movement.

This method is particularly valuable when a binocular microscope is used.

Miscellaneous remarks. Columns 4 and 5 in Table 1 are alternatives; so are columns 6 and 7. The phase-plate used in columns 5 and 6 is held very close to the back lens of the objective, and it is rather difficult to make a cell to hold it in this position. The cell for the phase-plate described in columns 4 and 7 is easier to make, but the results obtained are probably not quite so good.

On changing from a medium-power to a high-power objective, it is generally necessary to move the lamp, so as to get the correct distance between the illuminating annulus and the lower lens of this condenser. It will be found convenient to make marks on the bench, so that the lamp may be quickly placed in the right position.

Cleanliness is even more important than in ordinary microscopy. Any dirt on the top lens of the condenser *or on the bottom of the microscopical glass slide carrying the object* is likely to divert some of the direct light so that it falls elsewhere than on the annulus; this necessarily reduces contrast.

The preparation should be as thin as possible; that is to say, there should be no unnecessary fluid or mounting medium between slide and coverglass.

We claim for this method of phase-contrast microscopy that it is at least as good as the ordinary method, much less expensive, and applicable to any microscope.

ACKNOWLEDGEMENTS

Dr. O. L. Thomas's collaboration only came to an end when he left this country for New Zealand. We had already discussed with him our proposals for attempts to improve the method.

Professor A. C. Hardy, F.R.S., has continued to give every possible support to our work. We have received valuable advice on matters of theory from Dr. H. Kuhn and Dr. A. G. Oettlé. Mr. A. R. S. Bell has rendered us particularly skilful practical assistance.

SUMMARY

The following are the main improvements that we have made in the method of phase-contrast microscopy described by Kempson, Thomas, and Baker (1948):

1. No bull's-eye condenser is used. The illuminant is an electric bulb with a 'porcelain-processed', 'flashed white', or 'opal' surface.
2. No oiled paper is placed over the illuminating annulus.

3. The thickness of the deposit of magnesium fluoride on the phase-plate is controlled by observations on the interference colours given by surface reflections.

4. Positive (dark) phase-contrast is preferred for most purposes to negative (bright).

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The Cell-Theory: a Restatement, History, and Critique

Addendum to Part II

BY

JOHN R. BAKER

(*From the Department of Zoology and Comparative Anatomy, Oxford*)

SINCE writing Part II of this series of papers (Baker, 1949), I have had the good fortune to find a figure, published by Roffredi in 1775, which shows nuclei in the eggs and embryos of a nematode. This figure antedates by fifty-five years Purkinje's description of the germinal vesicle (1830) (or by fifty years, if we use the date of the private circulation of Purkinje's memoir).

Roffredi (1775) describes the anatomy of a small nematode obtained by wrapping flour-paste in linen and leaving the parcel in earth. It is evident from the drawing (Fig. 1 on the plate placed before p. 297) that the animal is a female belonging to the genus *Rhabditis*. The anterior and posterior ovaries and the eggs and young embryos are shown.

Roffredi regarded the whole of the female system as the 'ovaire ou matrice', and called the true ovaries (or ovaries and oviducts) its 'extrémités'. Nuclei are clearly represented in the oocytes that lie in a row along each ovary, and also in the eggs and embryos. Mutual pressure in the ovaries gives each of the oocytes a squarish appearance, and he calls the cell and its nucleus 'le quarré et le globule'. He does not give a written description of the nucleus in the mature egg nor in the embryo.

Spots are shown in some of the nuclei, but there can be no certainty that any of them represent nucleoli, and it seems best to continue to credit Fontana (1781) with the discovery of this organelle.

Roffredi unfortunately failed to distinguish the boundaries of the blastomeres. It is tantalizing to consider how easily he could have discovered cleavage if he had followed up his observations.

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[Quarterly Journal Microscopical Science, Vol. 90, part 3, September 1949.]

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Azo Eosin
Azo Fuchsin
Azur 1, 2, A, B, C, & L
Azur 2 Eosin
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Bromcresol Purple
Bromphenol Red
B.Z.L. Blue
Canada Balsam
Carbol Fuchsin
Carminic Acid
Cedarwood Oil
Celloidin
Chlorazol Black
Corollin
Crystal Violet
Dahlia
Dextrose
Dimethyl Yellow
Dioxane
D.P.X. Mountant
Dulcitol
Eosin
Eosin, Azur
Erythrosin
Evans Blue (Genuine)
Evans Blue (Substitute)
Fast Green, F.C.F.
Field's Stain
Formalin
Fuchsin
Gallamine Blue
Giemsa Stain
Gold Chloride
Haematein
Haematoxylin
Indian Ink, Micro
Indigo Carmine
Indophenol
Isamine (Pyrrol) Blue
Janus Black
Janus Blue
Janus Brown
Janus Green, B
Janus Red
Janus Yellow
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Leishman Stain
Magdala Red
Maltose
Mannite
May-Grunwald Stain
Metachrome Yellow 2RD
Methyl Green
Methyl Green-Orange G (KARDOS)
Methyl Green-Pyronin
Methyl Yellow
Methylene Blue
Methylene Blue (Vital)
Methylene Green
Methylene Violet (Berthsen)
Napthol Green
Neutral Red
Nile Blue
Nuclear Fast Red
Orange G
Orcein (Unna)
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Patent Blue
Peptones
Phenosafranin
Polyric Oil
Ponceau de Xylidine
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Rosolic Acid
Ruthenium Red
Safranin
Saffron
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Lison, L., 1936. *Histochimie Animale.* Paris (Gauthier-Villars).

Wigglesworth, V. B., 1933. Quart. J. micr. Sci., **76**, 270.

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Illustrations should be made about half as large again as the size required in print. Thus a drawing destined to occupy a whole page should be about 7½″ × 11″ (20 cm. × 30 cm.), including lettering.

Drawings should be 'line and dot' in black process ink. (The attention of authors is drawn to 'A Method of Illustration for Zoological Papers', by H. G. Cannon, published by the Association of British Zoologists, 1936.)

Photographs should not be given if a line and dot drawing will serve the purpose. Photographic prints should be sharp and with good contrast.

Lithographic and coloured illustrations will **not** be published unless the author is prepared to make a grant covering the cost of their production.

Position of Figures and Plates should be clearly indicated by notes written in the margin of the typescript.

The lettering of figures should be written in block capitals on accurately imposed outlines on tracing-paper. Abbreviations should not be used unless absolutely necessary. These remarks apply both to text-figures and to plates. The magnification of a figure is best indicated by a scale at the side of it.

Authors receive 50 copies of their contributions gratis, and may buy additional copies if they apply to the Editors when they return the corrected proofs.

Bodies of numerical data too extensive for complete publication should be summarized for discussion, and the originals deposited in the Archives established for this purpose in the British Museum (Natural History).